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(54) **ANTIGEN-BINDING MOLECULE CAPABLE OF BINDING TO TWO OR MORE ANTIGEN MOLECULES REPEATEDLY**

(57) The present inventors discovered that antibodies having weaker antigen-binding activity at the early endosomal pH in comparison with that at the pH of plasma are capable of binding to multiple antigen molecules

with a single antibody molecule, have long half-lives in plasma, and have improved durations of time in which they can bind to antigen.

**EP 2 275 443 A1**

## Description

## Technical Field

5 [0001] The present invention relates to methods for improving the pharmacokinetics of antigen-binding molecules and methods for increasing the number of times of antigen-binding of antigen-binding molecules, as well as antigen-binding molecules having improved pharmacokinetics, antigen-binding molecules having increased number of times of antigen-binding, and methods for producing such molecules.

## 10 Background Art

[0002] Antibodies are drawing attention as pharmaceuticals as they are highly stable in plasma and have few adverse effects. At present, a number of IgG-type antibody pharmaceuticals are available on the market and many more antibody pharmaceuticals are currently under development (Non-Patent Documents 1 and 2). Meanwhile, various technologies applicable to second-generation antibody pharmaceuticals have been developed, including those that enhance effector function, antigen-binding ability, pharmacokinetics, and stability, and those that reduce the risk of immunogenicity (Non-Patent Document 3). In general, the requisite dose of an antibody pharmaceutical is very high. This, in turn, has led to problems, such as high production cost, as well as the difficulty in producing subcutaneous formulations. In theory, the dose of an antibody pharmaceutical may be reduced by improving antibody pharmacokinetics or improving the affinity between antibodies and antigens.

[0003] The literature has reported methods for improving antibody pharmacokinetics using artificial substitution of amino acids in constant regions (Non-Patent Documents 4 and 5). Similarly, affinity maturation has been reported as a technology for enhancing antigen-binding ability or antigen-neutralizing activity (Non-Patent Document 6). This technology enables enhancement of antigen-binding activity by introduction of amino acid mutations into the CDR region of a variable region or such. The enhancement of antigen-binding ability enables improvement of *in vitro* biological activity or reduction of dosage, and further enables improvement of *in vivo* efficacy (Non-Patent Document 7).

[0004] The antigen-neutralizing capacity of a single antibody molecule depends on its affinity. By increasing the affinity, an antigen can be neutralized by smaller amount of an antibody. Various methods can be used to enhance the antibody affinity. Furthermore, if the affinity could be made infinite by covalently binding the antibody to the antigen, a single antibody molecule could neutralize one antigen molecule (a divalent antibody can neutralize two antigen molecules). However, the stoichiometric neutralization of one antibody against one antigen (one divalent antibody against two antigens) is the limit of pre-existing methods, and thus it is impossible to completely neutralize antigen with the smaller amount of antibody than the amount of antigen. In other words, the affinity enhancing effect has a limit (Non-Patent Document 9). To prolong the neutralization effect of a neutralizing antibody for a certain period, the antibody must be administered at a dose higher than the amount of antigen produced in the body during the same period. With the improvement of antibody pharmacokinetics or affinity maturation technology alone described above, there is thus a limitation in the reduction of the required antibody dose.

[0005] Accordingly, in order to sustain antibody's antigen-neutralizing effect for a target period with smaller amount of the antibody than the amount of antigen, a single antibody must neutralize multiple antigens. Methods for neutralizing multiple antigens with a single antibody include antigen inactivation using catalytic antibodies, which are antibodies conferred with a catalytic function. When the antigen is a protein, it can be inactivated by hydrolyzing its peptide bonds. An antibody can repeatedly neutralize antigens by catalyzing such hydrolysis (Non-Patent Document 8). There are many previous reports published on catalytic antibodies and technologies for producing them. However, there have been no reports of catalytic antibodies having sufficient catalytic activity as a pharmaceutical agent. Specifically, in an antibody *in vivo* study for a certain antigen, there has been no publication of catalytic antibodies which can produce a comparable or stronger effect even at low doses or produce a more prolonged effect even at a same dose as compared to an ordinary non-catalytic neutralizing antibody.

[0006] As described above, there have been no reports of antibodies that can produce a more superior *in vivo* effect than ordinary neutralizing antibodies through a single antibody neutralizing multiple antigen molecules. Thus, from the viewpoint of dose reduction and prolongation of the durability, there is a need for new technologies that permit the production of novel antibody molecules having a stronger *in vivo* effect than ordinary neutralizing antibodies by individually neutralizing multiple antigen molecules.

[0007] Prior art documents related to the present invention are shown below:

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[Prior art documents]

[Non-Patent Documents]

**[0008]**

Non-Patent Document 1: Monoclonal antibody successes in the clinic. Janice M Reichert, Clark J Rosensweig, Laura B Faden & Matthew C Dewitz, Nature Biotechnology 23, 1073-1078 (2005) Non-Patent Document 2: Pavlou AK, Belsey MJ. The therapeutic antibodies market to 2008. Eur J Pharm Biopharm. 2005 Apr;59(3):389-96

Non-Patent Document 3: Kim SJ, Park Y, Hong HJ. Antibody engineering for the development of therapeutic antibodies. Mol Cells. 2005 Aug 3 1;20(1):17-29. Review

Non-Patent Document 4: Hinton PR, Xiong JM, Johlfs MG, Tang MT, Keller S, Tsurushita N. An engineered human IgG1 antibody with longer serum half-life. J Immunol. 2006 Jan 1;176(1):346-56

Non-Patent Document 5: Ghetie V, Popov S, Borvak J, Radu C, Matesoi D, Medesan C, Ober RJ, Ward ES. Increasing the serum persistence of an IgG fragment by random mutagenesis. Nat Biotechnol. 1997 Jul;15(7):637-40

Non-Patent Document 6: Rajpal A, Beyaz N, Haber L, Cappuccilli G, Yee H, Bhatt RR, Takeuchi T, Lerner RA, Crea R. A general method for greatly improving the affinity of antibodies by using combinatorial libraries. Proc Natl Acad Sci USA. 2005 Jun 14;102(24):8466-71. Epub 2005 Jun 6

Non-Patent Document 7: Wu H, Pfarr DS, Johnson S, Brewah YA, Woods RM, Patel NK, White WI, Young JF, Kiener PA. Development of Motavizumab, an Ultra-potent Antibody for the Prevention of Respiratory Syncytial Virus Infection in the Upper and Lower Respiratory Tract. J Mol Biol. 2007, 368, 652-665

Non-Patent Document 8: Hanson CV, Nishiyama Y, Paul S. Catalytic antibodies and their applications. Curr Opin Biotechnol. 2005 Dec;16(6):631-6

Non-Patent Document 9: Rathanaswami P, Roalstad S, Roskos L, Su QJ, Lackie S, Babcook J. Demonstration of an in vivo generated sub-picomolar affinity fully human monoclonal antibody to interleukin-8. Biochem Biophys Res Commun. 2005 Sep 9;334(4):1004-13

#### Summary of the Invention

[Problems to be Solved by the Invention]

**[0009]** The above noted circumstances led to the discoveries of the present invention. Accordingly, an objective of the present invention is to provide methods for binding antigen-binding molecules to the antigens multiple times and methods for improving the pharmacokinetics of antigen-binding molecules, as well as antigen-binding molecules that are capable of binding to the antigens multiple times, antigen-binding molecules having improved pharmacokinetics, pharmaceutical compositions containing such antigen-binding molecules, and methods for producing such molecules and compositions.

[Means for Solving the Problems]

**[0010]** Dedicated studies on methods for binding polypeptides having antigen-binding ability, such as antigen-binding molecules, to the antigens multiple times, and methods for improving the half-lives of such molecules in plasma (blood) (improving their pharmacokinetics) were conducted herein. As a result, it was discovered that if the antigen-binding activity of an antigen-binding molecule at the early endosomal pH is lower than its antigen-binding activity at the pH of plasma (blood), it would be able to bind to antigens multiple times and have a longer half-life in plasma.

**[0011]** Accordingly, the present invention relates to methods for binding antigen-binding molecules to antigens multiple times, methods for improving the pharmacokinetics of antigen-binding molecules, and methods for producing antigen-binding molecules with improved pharmacokinetics; the present invention also relates to antigen-binding molecules that are capable of binding to antigens multiple times and antigen-binding molecules with improved pharmacokinetics. More specifically, the present invention provides:

- [1] an antigen-binding molecule having a  $KD(pH5.8)/KD(pH7.4)$  value, defined as the ratio of  $KD$  for the antigen at pH 5.8 and  $KD$  for the antigen at pH 7.4, of 2 or higher;
- [2] the antigen-binding molecule of [1], wherein the  $KD(pH5.8)/KD(pH7.4)$  value is 10 or higher;
- [3] the antigen-binding molecule of [1], wherein the  $KD(pH5.8)/KD(pH7.4)$  value is 40 or higher;
- [4] the antigen-binding molecule of any one of [1] to [3], wherein at least one amino acid of the antigen-binding molecule has been substituted with histidine, or at least one histidine has been inserted into the antigen-binding molecule;

[5] the antigen-binding molecule of any one of [1] to [4], wherein the antigen-binding molecule has an antagonistic activity;

[6] the antigen-binding molecule of any one of [1] to [5], wherein the antigen-binding molecule binds to a membrane antigen or a soluble antigen;

[7] the antigen-binding molecule of any one of [1] to [6], wherein the antigen-binding molecule is an antibody;

[8] a pharmaceutical composition comprising the antigen-binding molecule of any one of [1] to [7];

[9] a method for improving the pharmacokinetics of an antigen-binding molecule by impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4;

[10] a method for increasing the number of times of antigen-binding for an antigen-binding molecule by impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4;

[11] a method for increasing the number of antigens that can be bound by an antigen-binding molecule by impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4;

[12] a method for dissociating within a cell an antigen from an extracellularly-bound antigen-binding molecule by impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4;

[13] a method for releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an antigen-free form to the outside of the cell by impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4;

[14] a method for increasing the ability of an antigen-binding molecule to eliminate an antigen in plasma by impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4;

[15] the method of any one of [9] to [14], wherein the  $KD(pH5.8)/KD(pH7.4)$  value, defined as the ratio of  $KD$  for the antigen at pH 5.8 and  $KD$  for the antigen at pH 7.4, is 2 or higher;

[16] the method of any one of [9] to [14], wherein the  $KD(pH5.8)/KD(pH7.4)$  value is 10 or higher;

[17] the method of any one of [9] to [14], wherein the  $KD(pH5.8)/KD(pH7.4)$  value is 40 or higher;

[18] a method for improving the pharmacokinetics of an antigen-binding molecule by substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule;

[19] a method for increasing the number of times of antigen-binding for an antigen-binding molecule by substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule;

[20] a method for increasing the number of antigens that can be bound by an antigen-binding molecule by substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule;

[21] a method for dissociating within a cell an antigen from an extracellularly-bound antigen-binding molecule by substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule;

[22] a method for releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an antigen-free form to the outside of the cell, by substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule;

[23] a method for increasing the ability of an antigen-binding molecule to eliminate an antigen in plasma by substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule;

[24] the method of any one of [18] to [23], wherein the histidine substitution or insertion increases the  $KD(pH5.8)/KD(pH7.4)$  value, defined as the ratio of the antigen-binding activity at pH 5.8 and the antigen-binding activity at pH 7.4, as compared to the  $KD(pH5.8)/KD(pH7.4)$  value before the histidine substitution or insertion;

[25] the method of any one of [9] to [24], wherein the antigen-binding molecule has an antagonistic activity;

[26] the method of any one of [9] to [25], wherein the antigen-binding molecule binds to a membrane antigen or a soluble antigen;

[27] the method of any one of [9] to [26], wherein the antigen-binding molecule is an antibody;

[28] a method of screening for an antigen-binding molecules, which comprises the steps of:

(a) determining the antigen-binding activity of an antigen-binding molecule at pH 6.7 to pH 10.0;

(b) determining the antigen-binding activity of the antigen-binding molecule at pH 4.0 to pH 6.5; and

(c) selecting an antigen-binding molecule whose antigen-binding activity at pH 6.7 to pH 10.0 is greater than the antigen-binding activity at pH 4.0 to pH 6.5;

[29] the screening method of [28], which comprises the step of selecting an antibody whose antigen-binding activity at pH 6.7 to pH 10.0 is twice or higher that of the antigen-binding activity at pH 4.0 to pH 6.5;

[30] a method of screening for an antigen-binding molecule, which comprises the steps of:



- (a) binding an antigen-binding molecule to an antigen under a condition of pH 6.7 to pH 10.0;
- (b) placing the antigen-binding molecule that bound to the antigen of (a) under a condition of pH 4.0 to pH 6.5; and
- (c) obtaining an antigen-binding molecule that dissociated under the condition of pH 4.0 to pH 6.5;

[31] a method of screening for an antigen-binding molecule whose binding activity at a first pH is greater than that at a second pH, which comprises the steps of:

- (a) binding an antigen-binding molecule to an antigen-immobilized column under the condition of a first pH;
- (b) eluting the antigen-binding molecule that had bound to the column at the first pH from the column under the condition of a second pH; and
- (c) collecting the eluted antigen-binding molecule;

[32] a method of screening for an antigen-binding molecule whose binding activity at a first pH is greater than that at a second pH, which comprises the steps of:

- (a) binding an antigen-binding molecule library to an antigen-immobilized column under the condition of a first pH;
- (b) eluting the antigen-binding molecule from the column under the condition of a second pH;
- (c) amplifying the gene encoding the eluted antigen-binding molecule; and
- (d) obtaining the eluted antigen-binding molecule.

[33] the screening method of [31] or [32], wherein the first pH is 6.7 to 10.0 and the second pH is 4.0 to 6.5;

[34] the screening method of any one of [28] to [33], wherein at least one or more amino acids of the antigen-binding molecule has been substituted with histidine, or at least one histidine has been inserted into the antigen-binding molecule;

[35] the screening method of any one of [28] to [33], for obtaining an antigen-binding molecule that is superior in retention in the plasma;

[36] the screening method of any one of [28] to [33], for obtaining an antigen-binding molecule that is capable of binding to an antigen two or more times;

[37] the screening method of any one of [28] to [33], for obtaining an antigen-binding molecule that is capable of binding to more antigens as compared to the number of its antigen-binding sites;

[38] the screening method of any one of [28] to [33], for obtaining an antigen-binding molecule that dissociates an extracellularly-bound antigen within a cell.

[39] the screening method of any one of [28] to [33], for obtaining an antigen-binding molecule that is bound to an antigen and internalized into a cell, and released to the outside of the cell in an antigen-free form;

[40] the screening method of any one of [28] to [33], for obtaining an antigen-binding molecule that has increased ability to eliminate an antigen in plasma;

[41] the screening method of any one of [28] to [40], wherein the antigen-binding molecule is used as a pharmaceutical composition;

[42] the screening method of any one of [28] to [41], wherein the antigen-binding molecule is an antibody;

[43] a method for producing an antigen-binding molecule, which comprises the steps of:

- (a) determining the antigen-binding activity of an antigen-binding molecule at pH 6.7 to pH 10.0;
- (b) determining the antigen-binding activity of the antigen-binding molecule at pH 4.0 to pH 6.5;
- (c) selecting the antigen-binding molecule whose antigen-binding activity at pH 6.7 to pH 10.0 is greater than that at pH 4.0 to pH 6.5;
- (d) obtaining the gene encoding the antigen-binding molecule selected in (c); and
- (e) producing the antigen-binding molecule using the gene obtained in (d);

[44] a method for producing an antigen-binding molecule, which comprises the steps of:

- (a) binding an antigen-binding molecule to an antigen under a condition of pH 6.7 to pH 10.0;
- (b) allowing the antigen-binding molecule bound to the antigen of (a) to stand under a condition of pH 4.0 to pH 6.5;
- (c) collecting the antigen-binding molecule that dissociated under the condition of pH 4.0 to pH 6.5;
- (d) obtaining the gene encoding the antigen-binding molecule obtained in (c); and
- (e) producing the antigen-binding molecule using the gene obtained in (d);

[45] a method for producing an antigen-binding molecule whose binding activity at a first pH is greater than that at a second pH, which comprises the steps of:

- (a) binding an antigen-binding molecule to an antigen-immobilized column under the first pH condition;  
 (b) eluting the antigen-binding molecule, which is bound to the column at the first pH, from the column under a second pH condition;  
 (c) collecting the eluted antigen-binding molecule;  
 (d) obtaining the gene encoding the antigen-binding molecule obtained in (c); and  
 (e) producing the antigen-binding molecule using the gene obtained in (d);

[46] a method for producing an antigen-binding molecule whose binding activity at a first pH is greater than that at a second pH, which comprises the steps of:

- (a) binding an antigen-binding molecule library to an antigen-immobilized column under the first pH condition;  
 (b) eluting the antigen-binding molecule from the column under the second pH condition;  
 (c) amplifying the gene encoding the eluted antigen-binding molecule;  
 (d) collecting the eluted antigen-binding molecule;  
 (e) obtaining the gene encoding the antigen-binding molecule collected in (d); and  
 (f) producing the antigen-binding molecule using the gene obtained in (e);

[47] the production method of [45] or [46], wherein the first pH is 6.7 to 10.0 and the second pH is 4.0 to 6.5.

[48] the production method of any one of [43] to [47], which further comprises the step of substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule;

[49] the production method of any one of [43] to [48], wherein the antigen-binding molecule is an antibody;

[50] a pharmaceutical composition comprising an antigen-binding molecule produced by the production method of any one of [43] to [49].

#### [Effects of the Invention]

**[0012]** The present invention provides methods for making single antigen-binding molecules to repeatedly bind to multiple antigen molecules. When an antigen-binding molecule binds to multiple antigen molecules, the pharmacokinetics of the antigen-binding molecule can be improved and such molecule can exert more superior *in vivo* effects than those of ordinary antigen-binding molecules.

#### Brief Description of the Drawings

#### **[0013]**

FIG. 1 is a diagram depicting a degradation pathway of antibodies bound to membrane-bound antigen.

FIG. 2 is a diagram depicting a mechanism by which IgG molecules are salvaged by FcRn.

FIG. 3 is a schematic diagram depicting the re-binding of IgG molecules to new antigen following dissociation from membrane-bound antigen within endosomes.

FIG. 4 is a schematic diagram depicting the re-binding of IgG molecules to new antigen following dissociation from soluble antigen within endosomes.

FIG. 5 is a diagram depicting the process of panning using an antigen-immobilized column.

FIG. 6 presents graphs depicting the results of phage ELISA for clones acquired by column panning. The upper graph depicts WT and the lower graph depicts CL5.

FIG. 7 is a graph depicting the biological neutralization activity of pH-dependently-binding anti-IL-6 receptor antibodies.

FIG. 8 presents graphs depicting results of Biacore sensorgram for binding of pH-dependently-binding anti-IL-6 receptor antibodies to soluble IL-6 receptor at pH 7.4. The top graph depicts WT; the second graph from the top depicts H3pl/L73; the third graph from the top depicts H170/L82; and the bottom depicts CLH5/L73.

FIG. 9 presents graphs depicting results of Biacore sensorgram for binding of pH-dependently-binding anti-IL-6 receptor antibodies to soluble IL-6 receptor at pH 5.8. The top graph depicts WT; the second graph from the top depicts H3pl/L73; the third graph from the top depicts H170/L82; and the bottom graph depicts CLH5/L73.

FIG. 10 presents graphs depicting results of Biacore sensorgram for association (pH 7.4) and dissociation (pH 5.8) of pH-dependently-binding anti-IL-6 receptor antibodies to membrane-type IL-6 receptor. The top graph depicts WT; the second graph from the top depicts H3pl/L73; the third graph from the top depicts H170/L82; and the bottom graph depicts CLH5/L73.

FIG. 11 is a Biacore sensorgram indicating repeated binding of pH-dependently-binding anti-IL-6 receptor antibodies

to SR344.

FIG. 12 is a graph depicting the total amount of bound antigen in a repetitive binding experiment of pH-dependently-binding anti-IL-6 receptor antibodies to SR344.

FIG. 13 is a graph depicting time courses of antibody plasma concentrations of pH-dependently-binding anti-IL-6 receptor antibodies in human IL-6 receptor transgenic mice.

FIG. 14 is a graph depicting time courses of antibody plasma concentrations of pH-dependently-binding anti-IL-6 receptor antibodies in cynomolgus monkeys.

FIG. 15 is a graph depicting time courses of CRP concentrations in cynomolgus monkeys, in relation to pH-dependently-binding anti-IL-6 receptor antibodies.

FIG. 16 is a graph depicting time courses of unbound-type cynomolgus monkey IL-6 receptor concentrations in cynomolgus monkeys, in relation to pH-dependently-binding anti-IL-6 receptor antibodies.

FIG. 17 presents graphs depicting results of Biacore sensorgram of association (pH 7.4) and dissociation (pH 5.8) of pH-dependently-binding anti-IL-6 receptor antibodies to membrane-type IL-6 receptor. In order moving from the top, the results for WT, H3pl/L73-IgG1, Fv2-IgG1, and Fv4-IgG1 are shown.

FIG. 18 is a graph depicting time courses of plasma antibody concentrations of pH-dependently-binding anti-IL-6 receptor antibodies (WT, H3pl/L73-IgG1, Fv2-IgG1, and Fv4-IgG1) in human IL-6 receptor transgenic mice.

FIG. 19 presents graphs depicting results of Biacore sensorgram for association (pH 7.4) and dissociation (pH 5.8) of pH-dependently-binding anti-IL-6 receptor antibodies to membrane-type IL-6 receptor. Moving from the top down, the results for WT, Fv4-IgG1, Fv4-IgG2, and Fv4-M58 are shown.

FIG. 20 is a graph showing time courses of antibody plasma concentrations of pH-dependently-binding anti-IL-6 receptor antibodies (WT, Fv4-IgG1, Fv4-IgG2, and Fv4-M58) in human IL-6 receptor transgenic mice..

FIG. 21 presents graphs depicting results of Biacore sensorgram of association (pH 7.4) and dissociation (pH 5.8) of pH-dependently-binding anti-IL-6 receptor antibodies to membrane-type IL-6 receptor. Moving from the top down, the results for Fv1-M71, Fv1-M73, Fv3-M71, and Fv3-M73 are shown.

FIG. 22 is a graph depicting time courses of antibody plasma concentrations of pH-dependently-binding anti-IL-6 receptor antibodies in cynomolgus monkeys, during administration of H3pl/L73-IgG1, Fv1-M71, Fv1-M73, Fv2-IgG1, Fv3-M73, and Fv4-M73 at 0.5 mg/kg and during administration of high affinity Ab at 1.0 mg/kg.

FIG. 23 is a graph depicting time courses of CRP concentrations in cynomolgus monkeys, in relation to pH-dependently-binding anti-IL-6 receptor antibodies (H3pl/L73-IgG1-, Fv1-M71-, Fv1-M73-, Fv2-IgG1-, Fv3-M73-, Fv4-M73-, and high-affinity-Ab-administered groups).

FIG. 24 is a graph depicting time courses of unbound-type cynomolgus monkey IL-6 receptor concentrations in cynomolgus monkeys, in relation to pH-dependently-binding anti-IL-6 receptor antibodies (H3pl/L73-IgG1-, Fv1-M71-, Fv1-M73-, Fv2-IgG1-, Fv3-M73-, Fv4-M73-, and high-affinity-Ab-administered groups).

FIG. 25 is a diagram depicting FR1, FR2, FR3, and FR4 along with CDR1, CDR2, and CDR3 of heavy chains (VH1, VH2, VH3, VH4) and light chains (VL1, VL2, VL3). Asterisks indicate locations where amino acid mutations exist in the aligned sequences.

FIG. 26 presents a Biacore sensorgram depicting the pH-dependent binding of an anti-IL-6 antibody, Anti-IL6 clone 2, to IL-6 at pH 7.4 and pH 5.5. The curves in the sensorgram at pH 7.4 correspond to 100, 50, 25, 12.5, and 6.25 ng/mL IL-6, from above.

FIG. 27 presents a Biacore sensorgram depicting the pH-dependent binding of an anti-IL-31 receptor antibody, Anti-IL31R clone 1, to the IL-31 receptor at pH 7.4 and pH 5.5. The curves in the sensorgram at pH 5.5 correspond to 100, 50, 25, and 12.5 ng/mL IL-31 receptor, from above.

FIG. 28 depicts the time course of plasma antibody concentration after intravenous administration of a mixture solution containing SR344 and an anti-human IL-6 receptor antibody to mouse.

FIG. 29 depicts the time course of plasma SR344 concentration after intravenous administration of a mixture solution containing SR344 and an anti-human IL-6 receptor antibody to mouse.

#### Mode for Carrying Out the Invention

**[0014]** The present invention provides methods for increasing the number of times of antigen-binding in antigen-binding molecules. More specifically, the present invention provides methods for increasing the number of times of antigen-binding in antigen-binding molecules by impairing the antigen-binding ability of the antigen-binding molecules at acidic pH as compared to that at neutral pH. Furthermore, the present invention provides methods for increasing the number of times of antigen-binding in antigen-binding molecules by substituting histidine for at least one amino acid in the antigen-binding molecules or inserting at least one histidine into the antigen-binding molecules. In addition, the present invention provides methods for increasing the number of times of antigen-binding in antigen-binding molecules by substituting, deleting, adding, and/or inserting amino acids in the antibody constant region of antigen-binding molecules.

**[0015]** The present invention also provides methods for increasing the number of antigens that can be bound by an

antigen-binding molecule. More specifically, the present invention provides methods for increasing the number of antigens that can be bound by an antigen-binding molecule by impairing the antigen-binding ability at acidic pH as compared to that at neutral pH. Furthermore, the present invention provides methods for increasing the number of antigens that can be bound by an antigen-binding molecule by substituting histidine for at least one amino acid in the antigen-binding molecules or inserting at least one histidine into the antigen-binding molecules. In addition, the present invention provides methods for increasing the number of antigens that can be bound by an antigen-binding molecule through substituting, deleting, adding, and/or inserting amino acids in the antibody constant region of antigen-binding molecules.

**[0016]** The present invention also provides methods for dissociating within a cell an antigen from an extracellularly-bound antigen-binding molecule. More specifically, the present invention provides methods for dissociating within a cell an antigen from an extracellularly-bound antigen-binding molecule by impairing the antigen-binding ability at acidic pH as compared to that at neutral pH. Furthermore, the present invention provides methods for dissociating within a cell an antigen from an extracellularly-bound antigen-binding molecule by substituting histidine for at least one amino acid in the antigen-binding molecule or inserting at least one histidine into the antigen-binding molecule. In addition, the present invention provides methods for dissociating within a cell an antigen from an extracellularly-bound antigen-binding molecule through substituting, deleting, adding, and/or inserting amino acids in the antibody constant region of antigen-binding molecule.

**[0017]** The present invention also provides methods for releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an antigen-free form to the outside of the cell. More specifically, the present invention provides methods for releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an antigen-free form to the outside of the cell, by impairing the antigen-binding ability at acidic pH as compared to that at neutral pH. Furthermore, the present invention provides methods for releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an antigen-free form to the outside of the cell, by substituting histidine for at least one amino acid in the antigen-binding molecule or inserting at least one histidine into the antigen-binding molecule. In addition, the present invention provides methods for releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an antigen-free form to the outside of the cell, through substituting, deleting, adding, and/or inserting amino acids in the antibody constant region of antigen-binding molecule.

**[0018]** The present invention also provides methods for increasing the ability of an antigen-binding molecule to eliminate antigens in plasma. More specifically, the present invention provides methods for increasing the ability of an antigen-binding molecule to eliminate antigens in plasma by impairing the antigen-binding ability at acidic pH as compared to that at neutral pH. Furthermore, the present invention provides methods for increasing the ability of an antigen-binding molecule to eliminate antigens in plasma by substituting histidine for at least one amino acid in the antigen-binding molecules or inserting at least one histidine into the antigen-binding molecules. In addition, the present invention provides methods for increasing the ability of an antigen-binding molecule to eliminate antigens in plasma through substituting, deleting, adding, and/or inserting amino acids in the antibody constant region of antigen-binding molecule.

**[0019]** The present invention also provides methods for improving the pharmacokinetics of antigen-binding molecules. More specifically, the present invention provides methods for improving the pharmacokinetics of antigen-binding molecules (prolonging the retention in plasma) by impairing the antigen-binding ability at acidic pH as compared to that at neutral pH. Furthermore, the present invention provides methods for improving the pharmacokinetics of antigen-binding molecules by substituting histidine for at least one amino acid in the antigen-binding molecules or inserting at least one histidine into the antigen-binding molecules. In addition, the present invention provides methods for improving the pharmacokinetics of antigen-binding molecules by substituting, deleting, adding, and/or inserting amino acids in the antibody constant region of antigen-binding molecules.

**[0020]** Further, the present invention provides methods for increasing the ability of the antigen-binding molecules to eliminate antigens in plasma. More specifically, the present invention provides methods for increasing the ability of the antigen-binding molecules to eliminate antigens in plasma by impairing the antigen-binding ability of the antigen-binding molecules at acidic pH as compared to that at neutral pH. Furthermore, the present invention provides methods for increasing the ability of the antigen-binding molecules to eliminate antigens in plasma by substituting at least one amino acid in the antigen-binding molecules with histidine or inserting at least one histidine into the antigen-binding molecules. In addition, the present invention provides methods for increasing the ability of the antigen-binding molecules to eliminate antigens in plasma by substituting, deleting, adding, and/or inserting amino acids in the antibody constant region of antigen-binding molecules.

**[0021]** Herein, "improvement of the pharmacokinetics", "amelioration of the pharmacokinetics", "superior pharmacokinetics" are interchangeable with "improvement of the retention in plasma (blood)", "amelioration of the retention in plasma (blood)", and "superior retention in plasma (blood)", respectively, and these phrases are synonymous.

**[0022]** Herein, impairing the antigen-binding activity at acidic pH as compared to that at neutral pH means that the antigen-binding ability of an antigen-binding molecule at pH 4.0 to pH 6.5 is impaired as compared to that at pH 6.7 to pH 10.0, preferably that the antigen-binding activity of an antigen-binding molecule at pH 5.5 to pH 6.5 is impaired as

compared to that at pH 7.0 to pH 8.0, and more preferably that the antigen-binding activity of an antigen-binding molecule at pH 5.8 is impaired as compared to that at pH 7.4. Accordingly, in the present invention, acidic pH is typically pH 4.0 to pH 6.5, preferably pH 5.5 to pH 6.5, and more preferably pH 5.8. Alternatively, in the present invention, neutral pH is typically pH 6.7 to pH 10.0, preferably pH 7.0 to pH 8.0, and more preferably pH 7.4.

**[0023]** Herein, the phrase "impairing the antigen-binding ability of an antigen-binding molecule at acidic pH as compared to that at neutral pH" is interchangeable with the phrase "increasing the antigen-binding ability of an antigen-binding molecule at neutral pH as compared to that at acidic pH". In other words, in the present invention, the difference in the antigen-binding ability of an antigen-binding molecule should be increased between acidic and neutral pHs. For example, the value of  $KD(pH5.8)/KD(pH7.4)$  should be increased, as described below. The difference in the antigen-binding ability of an antigen-binding molecule between acidic and neutral pHs may be increased, for example, by either or both, impairing the antigen-binding ability at acidic pH and increasing the antigen-binding ability at neutral pH.

**[0024]** Conditions other than the pH for determining the antigen-binding activity can be selected appropriately by those skilled in the art, and the conditions are not particularly limited. The antigen-binding activity can be determined, for example, under conditions of MES buffer and 37°C as described in the Examples herein. Furthermore, the antigen-binding activity of an antigen-binding molecule can be determined by methods known to those skilled in the art, for example, using Biacore (GE Healthcare) or the like, as described in the Examples herein. When the antigen is a soluble antigen, the activity of binding to the soluble antigen can be assessed by injecting the antigen as an analyte onto a chip immobilized with the antigen-binding molecule. Alternatively, when the antigen is a membrane antigen, the activity of binding to the membrane antigen can be assessed by injecting the antigen-binding molecule as an analyte onto an antigen-immobilized chip.

**[0025]** In the present invention, the difference in the antigen-binding activity between acidic and neutral pHs is not particularly as long as the antigen-binding activity at acidic pH is lower than that at neutral pH. However, the value of  $KD(pH5.8)/KD(pH7.4)$ , which is a ratio of dissociation constant (KD) against an antigen at pH 5.8 and that at pH 7.4, is preferably 2 or greater, more preferably 10 or greater, and still more preferably 40 or greater. The upper limit of  $KD(pH5.8)/KD(pH7.4)$  value is not particularly limited, and may be any value, for example, 400, 1,000, or 10,000, as long as the molecule can be produced by technologies of those skilled in the art. When the antigen is a soluble antigen, the antigen-binding activity can be presented in terms of the dissociation constant (KD). Alternatively, when the antigen is a membrane antigen, the antigen-binding activity can be presented in terms of the apparent dissociation constant. The dissociation constant (KD) and apparent dissociation constant (apparent KD) can be determined by methods known to those skilled in the art, for example, using Biacore (GE healthcare), Scatchard plot, or FACS.

**[0026]** Alternatively, it is possible to use, for example,  $k_d$ , a dissociation rate constant, as an indicator for the difference in the antigen-binding activity between acidic and neutral pHs. When the dissociation rate constant ( $k_d$ ) is used as an indicator for the difference in the binding activity instead of the dissociation constant (KD), the value of  $k_d(pH5.8)/k_d(pH7.4)$ , which is a ratio of dissociation rate constant ( $k_d$ ) against an antigen at pH 5.8 and that at pH 7.4, is preferably 2 or greater, more preferably 5 or greater, even more preferably 10 or greater, and still more preferably 30 or greater. The upper limit of  $k_d(pH5.8)/k_d(pH7.4)$  value is not particularly limited, and may be any value, for example, 50, 100, or 200, as long as the molecule can be produced by technologies common to those skilled in the art.

**[0027]** When the antigen is a soluble antigen, the antigen-binding activity can be presented in terms of the dissociation rate constant ( $k_d$ ). Alternatively, when the antigen is a membrane antigen, the antigen-binding activity can be presented in terms of the apparent dissociation rate constant. The dissociation rate constant ( $k_d$ ) and apparent dissociation rate constant (apparent  $k_d$ ) can be determined by methods known to those skilled in the art, for example, using Biacore (GE healthcare) or FACS.

**[0028]** In the present invention, when the antigen-binding activity of an antigen-binding molecule is determined at different pHs, it is preferred that the measurement conditions except for pH are constant.

**[0029]** The methods for impairing the antigen-binding activity of an antigen-binding molecule at pH 5.8 as compared to that at pH 7.4 (methods for conferring the pH-dependent binding ability) are not particularly limited and may be any methods. Such methods include, for example, methods for impairing the antigen-binding activity at pH 5.8 as compared to that at pH 7.4 by substituting histidine for amino acids in the antigen-binding molecule or inserting histidine into the antigen-binding molecule. It is already known that an antibody can be conferred with a pH-dependent antigen-binding activity by substituting histidine for amino acids in the antibody (FEBS Letter, 309(1), 8588 (1992)). Such histidine mutation (substitution) or insertion sites are not particularly limited, and any site is acceptable as long as the antigen-binding activity at pH 5.8 is lowered than that at pH 7.4 (the value of  $KD(pH5.8)/KD(pH7.4)$  gets greater) as compared to before mutation or insertion. When the antigen-binding molecule is an antibody, such sites include, for example, sites within an antibody, variable region. The appropriate number of histidine mutation or insertion sites can be appropriately determined by those skilled in the art. Histidine may be substituted or inserted at a single site, or two or more sites. It is also possible to introduce non-histidine mutation (mutation with amino acids other than histidine) at the same time. Furthermore, histidine mutation may be introduced simultaneously with histidine insertion. It is possible to substitute or insert histidine at random using a method such as histidine scanning, which uses histidine instead of alanine in alanine



scanning known to those skilled in the art. Alternatively, antigen-binding molecules whose  $KD(pH5.8)/KD(pH7.4)$  is increased as compared to before mutation can be selected from an antigen-binding molecule library with random histidine mutation or insertion.

**[0030]** When histidine is substituted for amino acids of an antigen-binding molecule or inserted between amino acids of the molecule, it is preferred, but not required, that the antigen-binding activity of the antigen-binding molecule at pH 7.4 after histidine substitution or insertion is comparable to that at pH 7.4 before histidine substitution or insertion. The "antigen-binding activity of the antigen-binding molecule at pH 7.4 after histidine substitution or insertion is comparable to that at pH 7.4 before histidine substitution or insertion" means that even after histidine substitution or insertion, the antigen-binding molecule retains 10% or more, preferably 50% or more, more preferably 80% or more, and still more preferably 90% or more of the antigen-binding activity of before histidine substitution or insertion. When the antigen-binding activity of the antigen-binding molecule has been impaired due to histidine substitution or insertion, the antigen-binding activity may be adjusted by introducing substitution, deletion, addition, and/or insertion of one or more amino acids into the antigen-binding molecule so that the antigen-binding activity becomes comparable to that before histidine substitution or insertion. The present invention also includes such antigen-binding molecules having a comparable binding activity as a result of substitution, deletion, addition, and/or insertion of one or more amino acids after histidine substitution or insertion.

**[0031]** Alternative methods for impairing the antigen-binding activity of an antigen-binding molecule at pH 5.8 as compared to that at pH 7.4 include methods of substituting non-natural amino acids for amino acids in an antigen-binding molecule or inserting non-natural amino acids into amino acids of an antigen-binding molecule. It is known that the  $pK_a$  can be artificially controlled using non-natural amino acids (Angew. Chem. Int. Ed. 2005, 44, 34; Chem Soc Rev. 2004 Sep 10;33(7):422-30; Amino Acids. 1999;16(3-4):345-79). Thus, in the present invention, non-natural amino acids can be used instead of histidine described above. Such non-natural amino acid substitution and/or insertion may be introduced simultaneously with the histidine substitution and/or insertion described above. Any non-natural amino acids may be used in the present invention. It is possible to use non-natural amino acids known to those skilled in the art.

**[0032]** Furthermore, when the antigen-binding molecule is a substance having an antibody constant region, alternative methods for impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4 include methods for modifying the antibody constant region contained in the antigen-binding molecule. Such methods for modifying the antibody constant region include, for example, methods for substituting a constant region described in the Examples herein.

**[0033]** Alternative methods for modifying the antibody constant region include, for example, methods to assess various constant region isotypes (IgG1, IgG2, IgG3, and IgG4) and select an isotype that impairs the antigen-binding activity at pH 5.8 (increases the dissociation rate at pH 5.8). Alternatively, methods include those for impairing the antigen-binding activity at pH 5.8 (increasing the dissociation rate at pH 5.8) by substituting amino acids in the amino acid sequence of a wild-type isotype (the amino acid sequence of wild type IgG1, IgG2, IgG3, or IgG4). The sequence of the hinge region of an antibody constant region is considerably different among isotypes (IgG1, IgG2, IgG3, and IgG4), and the difference in the hinge region amino acid sequence has a great impact on the antigen-binding activity. Thus, it is possible to select an appropriate isotype to impair the antigen-binding activity at pH 5.8 (to increase the dissociation rate at pH 5.8) by considering the type of antigen or epitope. Furthermore, since the difference in the hinge region amino acid sequence has a significant influence on the antigen-binding activity, preferred amino acid substitution sites in the amino acid sequence of a wild type isotype are assumed to be within the hinge region.

**[0034]** When the antigen-binding activity of the antigen-binding substance at pH 5.8 is weakened compared to that at pH 7.4 (when  $KD(pH5.8)/KD(pH7.4)$  value is increased) by the above described methods and such, it is generally preferable that the  $KD(pH5.8)/KD(pH7.4)$  value be two times or more, more preferably five times or more, and even more preferably ten times or more as compared to that of the original antibody, although the invention is not particularly limited thereto.

**[0035]** Herein, the "improvement of the pharmacokinetics" means prolongation of the time required for the elimination of the antigen-binding molecule from plasma (for example, reaching the state where the antigen-binding molecule cannot return to the plasma due to degradation in cells, or other reasons) after administration, to an animal such as human, mouse, rat, monkey, rabbit, or dog, as well as prolongation of the plasma retention time of the antigen-binding molecule being in a form capable of binding to antigens (for example, being in an antigen-free form) during the period until it is eliminated from the plasma after administration. Even if an antigen-binding molecule is circulated in plasma, it cannot bind to an antigen when it already binds to another antigen. Accordingly, the period where the antigen-binding molecule can newly binds to another antigen is prolonged (the chance to bind another antigen increases) by prolonging the period where the antigen-binding molecule is in an antigen-free form. This makes it possible to shorten the period where the antigen is free from antigen-binding molecules *in vivo* (in other words, to prolong the period where the antigen is bound by an antigen-binding molecule). For example, the ratio of antigens bound to antigen-binding molecules against the antigens in the body in plasma (total of antigen molecules bound to and free from the antigen-binding molecules) generally decreases in a certain period of time after the administration of the antigen-binding molecules. However, such decrease

can be suppressed (for example, the degree of decrease can be made smaller) by prolonging the retention time of the antigen-binding molecules in a form capable of binding to antigens. This results in an increase in the ratio of antigens bound to antigen-binding molecules against the antigens in the body in a certain period of time after antibody administration.

**[0036]** Specifically, in the present invention, the "improvement of the pharmacokinetics" does not necessarily mean the prolongation (extension) of the time required for the elimination of the antigen-binding molecule after administration. Even if the time required for the elimination of the antigen-binding molecule after administration remains unchanged, the pharmacokinetics can be said "improved" in the present invention if the plasma retention time of the antigen-binding molecule being in a form capable of binding to an antigen (for example, the antigen-binding molecule being in an antigen-free form) is prolonged; the period where the antigen is free from an antigen-binding molecule in the body is shortened (in other words, the period where the antigen-binding molecule is bound to an antigen is prolonged); and the ratio of antigens bound to antigen-binding molecules against the antigens in the body is increased. Thus, in the present invention, the "improvement of the pharmacokinetics" encompasses at least:

- (1) prolongation of the time required for the elimination of the antigen-binding molecule from plasma after administration of the antigen-binding molecule;
- (2) prolongation of the plasma retention time of the antigen-binding molecule in a form capable of binding to an antigen after administration of the antigen-binding molecule;
- (3) shortening of the period where the antigen is free from an antigen-binding molecule in the body after administration of the antigen-binding molecule (prolongation of the period where the antigen-binding molecule is bound to an antigen in the body); and
- (4) increase in the ratio of antigens bound to antigen-binding molecules to the antigen in the body.

**[0037]** When the antigen is a soluble antigen present in plasma, even if the pharmacokinetics of the antigen-binding molecule (rate of elimination from plasma) is equivalent, there are cases where elimination of antigen bound to the antigen-binding molecule is accelerated. Reducing the pharmacokinetics of the antigen (accelerating elimination from plasma) results in the relative improvement of the pharmacokinetics of the antigen-binding molecule, and thus, leads to the prolongation of the time of the antigen-binding molecule present in plasma in a form capable of binding to antigens. Thus, in one embodiment, the "improvement of the pharmacokinetics" of antigen-binding molecules of the present invention includes increasing the rate of eliminating soluble antigens from plasma after administration of the antigen-binding molecules (the ability of the antigen-binding molecule to eliminate antigens from plasma).

**[0038]** In the present invention, when the antigen is a membrane antigen, whether a single antigen-binding molecule binds to multiple antigens can be assessed by testing whether the pharmacokinetics of the antigen-binding molecule is improved. Whether the "pharmacokinetics is improved" can be assessed by the following method. For example, whether the time required for the elimination of an antigen-binding molecule after administration is prolonged can be assessed by determining any one of parameters for the antigen-binding molecule, such as half-life in plasma, mean plasma retention time, and clearance in plasma ("Pharmacokinetics: Enshu-niyoru Rikai (Understanding through practice)" Nanzando). For example, when the half-life in plasma or mean plasma retention time of an antigen-binding molecule administered to mice, rats, monkeys, rabbits, dogs, humans, or other animals is prolonged, the pharmacokinetics of the antigen-binding molecule is judged to be improved. These parameters can be determined by methods known to those skilled in the art. For example, the parameters can be appropriately assessed by noncompartmental analysis using pharmacokinetics analysis software WinNonlin (Pharsight) according to the appended instruction manual.

**[0039]** Alternatively, whether the plasma retention time of an antigen-binding molecule in a form capable of binding to antigens after administration of the antigen-binding molecule is prolonged can be assessed by measuring the plasma concentration of the antigen-free antigen-binding molecule and determining any one of parameters for the antigen-free antigen-binding molecule, such as half-life in plasma, mean plasma retention time, and clearance in plasma. The concentration of the antigen-free antigen-binding molecule in plasma can be measured by methods known to those skilled in the art. For example, such measurements are described in Clin Pharmacol. 2008 Apr;48(4):406-17.

**[0040]** Furthermore, whether the period where an antigen is free from the antigen-binding molecules in the body after administration of the antigen-binding molecules is shortened (the period where the antigen-binding molecule is bound to an antigen in the body is prolonged) can be assessed by determining the plasma concentration of the unbound antigen that is free from antigen-binding molecules, and considering the period where the concentration of free antigen in plasma or the amount ratio of free antigen against the total antigen remains low. The plasma concentration of the free antigen or amount ratio of free antigen against total antigen can be determined by methods known to those skilled in the art. For example, such measurements are described in Pharm Res. 2006 Jan;23(1):95-103. Alternatively, when the antigen exerts some function *in vivo*, whether the antigen is bound by an antigen-binding molecule that neutralizes the antigen's function (antagonistic molecule) can be assessed by testing whether the function of the antigen is neutralized. Whether



the function of the antigen is neutralized can be assessed by assaying an *in vivo* marker that reflects the function of the antigen. Whether the antigen is bound by an antigen-binding molecule that activates the function of the antigen (agonistic molecule) can be assessed by assaying an *in vivo* marker that reflects the function of the antigen.

[0041] There is no particular limitation on the determination of the plasma concentration of free antigen and amount ratio of free antigen against total antigen, and *in vivo* marker assay, but the determination is preferably carried out after a certain period following administration of an antigen-binding substance. In the present invention, such a period following administration of an antigen-binding substance is not particularly limited, and an appropriate period can be determined by those skilled in the art depending on the properties of the administered antigen-binding substance and the like. Examples of the period are: one day after administration of an antigen-binding substance; three days after administration of an antigen-binding substance, seven days after administration of an antigen-binding substance, 14 days after administration of an antigen-binding substance, and 28 days after administration of an antigen-binding substance.

[0042] In the present invention, it is preferred to improve the pharmacokinetics in human. Even when the plasma retention in human is difficult to determine, it can be predicted based on the plasma retention in mice (for example, normal mice, human antigen-expressing transgenic mice, and human FcRn-expressing transgenic mice) or monkeys (for example, cynomolgus monkeys).

[0043] Methods for determining the retention in plasma are not particularly limited. The determination can be carried out, for example, according to the methods described in the Examples herein.

[0044] Whether an antigen-binding molecule is capable of binding to antigens multiple times can be assessed by testing whether the antigen bound to an antigen-binding molecule under the same neutral condition as plasma dissociates under the same acidic condition as endosome and how many antigens the antigen-binding molecule can rebind to under the neutral condition. Specifically, the assessment can be carried out by allowing the antigen-binding molecule and antigen to form a complex under the neutral condition, exposing the complex to an acidic condition for a predetermined period, and then testing whether the antigen-binding molecule can rebind to an antigen under the neutral condition, using a device for assaying antigen-binding molecule-antigen reactions, such as Biacore. When the antigen-binding capacity of the antigen-binding molecule conferred with the pH-dependent binding ability has been improved to twice that of the antigen-binding molecule before modification, the number of times of binding of the antigen-binding molecule conferred with the pH-dependent binding ability can be judged to be increased to twice that of the antigen-binding molecule before modification. Alternatively, when the antigen is a membrane antigen and thus the antigen-binding molecule is eliminated from plasma through antigen-mediated uptake and degradation in a lysosome, whether the number of times of binding of the antigen-binding molecule conferred with the pH-dependent binding ability is increased as compared to that before modification can be assessed by comparing the pharmacokinetics or duration of antigen binding between the antigen-binding molecule conferred with the pH-dependent binding ability and the antigen-binding molecule before modification. For example, when the antigen-binding duration of the antigen-binding molecule conferred with the pH-dependent binding ability is prolonged twice that of the antigen-binding molecule before modification, the number of times of binding of the antigen-binding molecule conferred with the pH-dependent binding ability is judged to be increased to twice that of the antigen-binding molecule before modification. Alternatively, when the plasma concentration of an unbound antigen, which is free from the antigen-binding molecule, is determined and the period where the plasma concentration of the free antigen or the amount ratio of the free antigen against the total antigen remains low is prolonged to twice, the number of times of binding of the antigen-binding molecule conferred with the pH-dependent binding ability is judged to be increased to twice that of the antigen-binding molecule before modification.

[0045] When the antigen is a soluble antigen, if the antigen bound to an antigen-binding molecule under the neutral condition in plasma dissociates in an endosome, and the antigen-binding molecule returns to the plasma, the antigen-binding molecule can again bind to an antigen under the neutral condition in plasma. Thus, an antigen-binding molecule that has the characteristics to dissociate with an antigen in acidic condition of an endosome is capable of binding to antigens multiple times. Compared to when the antigen bound to an antigen-binding molecule does not dissociate in an endosome (the antigen remains bound to the antigen-binding molecule when returning to plasma), when the antigen bound to an antigen-binding molecule dissociates in endosomes, the antigen is delivered to a lysosome and then degraded, and thus, the rate of elimination of the antigen from plasma increases. That is, it is also possible to determine whether the antigen-binding molecule is capable of binding to antigens multiple times using the rate of elimination of antigen from plasma as an index. The rate of elimination of the antigen from plasma can be determined, for example, by administering the antigens (e.g., membrane antigen) and antigen-binding molecules *in vivo*, and then measuring the concentration of antigens in plasma. When an antigen (e.g., membrane antigen) is produced or secreted *in vivo*, the plasma antigen concentration is reduced if the rate of elimination of the antigen from plasma is increased. Thus, it is also possible to determine whether the antigen-binding molecule is capable of binding to antigens multiple times using the plasma antigen concentration as an index.

[0046] Herein, "increasing the number of times of antigen-binding of the antigen-binding molecule" means that the number of cycles is increased when taking as one cycle the process where an antigen-binding molecule administered to human, mouse, monkey, or such binds to an antigen and is internalized into a cell. Specifically, herein, "the antigen-

binding molecule binds twice to an antigen" means that the antigen-binding molecule bound by an antigen is internalized into a cell and released in an antigen-free form to the outside of the cell, and the released antigen-binding molecule rebinds to another antigen and is internalized into a cell again.

**[0047]** When the antigen-binding molecule is internalized into a cell, it may be in a form bound by a single antigen, or two or more antigens.

**[0048]** Herein, "the number of times of antigen-binding of an antigen-binding molecule is increased" does not necessarily mean that the number of times of antigen-binding increases in every antigen-binding molecules. For example, among antigen-binding molecules in an antigen-binding-molecule composition, the proportion of antigen-binding molecules that bind to antigens twice or more times may increase, or the average number of binding events of antigen-binding molecules in an antigen-binding-molecule composition may increase.

**[0049]** In the present invention, it is preferred that the number of times of antigen-binding of an antigen-binding molecule increases when the molecule is administered to a human. However, when it is difficult to determine the number of times of antigen-binding in human, the number in human may be predicted based on the results obtained by *in vitro* assay or measurement using mice (for example, antigen-expressing transgenic mice and human FcRn-expressing transgenic mice) or monkeys (for example, cynomolgus monkeys).

**[0050]** In the present invention, it is preferred that an antigen-binding molecule binds to antigens twice or more times. For example, it is preferred that, of the antigen-binding molecules in an antigen-binding-molecule composition, at least 10% or more, preferably 30% or more, more preferably 50% or more, and still more preferably 80% or more (for example, 90% or more, 95% or more, and so on) bind to antigens twice or more times.

**[0051]** Herein, "increasing the number of antigens that can be bound by an antigen-binding molecule" means increasing the number of antigens that can be bound by an antigen-binding molecule during the period until the antigen-binding molecule is degraded in a lysosome of a cell after administration of the antigen-binding molecule to an animal such as human, mouse, or monkey.

**[0052]** In general, antibodies such as IgG have two binding domains, and thus a single antibody binds to a maximum of two antigens. An antibody bound to antigen(s) is internalized into a cell, and the antibody and antigen(s) are degraded in a lysosome. In general, antibodies such as IgG can bind to a maximum of two antigens. When the antigen-binding activity of an antigen-binding molecule such as an antibody at the endosomal pH is impaired as compared to that at the plasma pH by the methods of the present invention, the antigen-binding molecule such as an antibody internalized into a cell dissociates the antigen and is released to the outside of the cell, and thus can bind to another antigen again. In other words, the methods of the present invention enable for an antigen-binding molecule to bind to more antigens than the number of its antigen-binding sites. Specifically, by using the methods of the present invention, for example, IgG having two antigen-binding sites can bind to three or more antigens, preferably four or more antigens, during a period until the antibody is degraded after administration. For example, when the antibody is a neutralizing antibody, "increasing the number of antigens that can be bound by an antigen-binding molecule" is interchangeable with "increasing the number of antigens that the antigen-binding molecule can neutralize". Thus, "bind" can be replaced with "neutralize" when the antibody is a neutralizing antibody.

**[0053]** In the present invention, "increasing the number of antigens that can be bound by an antigen-binding molecule" does not necessarily mean increasing the number of antigens that can be bound by every antigen-binding molecule. For example, the average number of antigens that can be bound by an antigen-binding molecule in an antigen-binding-molecule composition may increase, or the proportion of antigen-binding molecules that can bind to more antigens than the number of their antigen-binding sites may increase.

**[0054]** In the present invention, it is preferred that the number of antigens that can be bound by an antigen-binding molecule increases when the molecule is administered to a human. However, when it is difficult to determine the number in human, it may be predicted based on the results obtained by *in vitro* assay or measurement using mice (for example, antigen-expressing transgenic mice and human FcRn-expressing transgenic mice) or monkeys (for example, cynomolgus monkeys). When the antibody is a neutralizing antibody, the above-described number of times of antigen-binding of the antigen-binding molecule is generally assumed to correlate with the number of antigens which can be neutralized by an antigen-binding molecule. Thus, the number of antigens which can be neutralized by an antigen-binding molecule can be determined by the same methods described above for determining the number of times of binding of an antigen-binding molecule.

**[0055]** Furthermore, the present invention provides methods for binding an antigen-binding molecule to antigens twice or more times in the body, by administering an antigen-binding molecule whose antigen-binding activity at acidic pH is lower than that at neutral pH.

**[0056]** The present invention also relates to methods for neutralizing antigens that are greater in number than the number of antigen-binding sites of an antigen-binding molecule having the neutralizing activity, by administering the antigen-binding molecule whose antigen-binding activity at acidic pH is lower than that at neutral pH. Preferably, the present invention relates to methods for neutralizing three or more antigens, preferably four or more antigens by administering IgG whose antigen-binding activity at acidic pH is lower than that at neutral pH.

[0057] The present invention also relates to methods for dissociating within a cell an antigen from an extracellularly-bound antigen-binding molecule by impairing the antigen-binding ability of the antigen-binding molecule at acidic pH as compared to that at neutral pH. In the present invention, the antigen may be dissociated from the antigen-binding molecule anywhere within a cell; however, it is preferred that the antigen is dissociated within an early endosome. In the present invention, "an antigen is dissociated within a cell from an extracellularly-bound antigen-binding molecule" does not necessarily mean that every antigen internalized into a cell via binding to the antigen-binding molecule is dissociated from the antigen-binding molecule within the cell. It is acceptable that the proportion of antigen that is dissociated from the antigen-binding molecule within a cell increases when compared to before impairing the antigen-binding ability of the antigen-binding molecule at acidic pH as compared to that at neutral pH.

[0058] Furthermore, the present invention relates to methods for enhancing the intracellular binding of an antigen-binding molecule free from an antigen to FcRn by impairing the antigen-binding ability of the antigen-binding molecule at acidic pH as compared to that at neutral pH. In general, FcRn binds to an antigen-binding molecule within an endosome. However, an antigen-binding molecule bound to a membrane antigen is assumed not to bind to FcRn. Thus, in a preferred embodiment, when the antigen is a membrane-bound antigen, the present invention includes methods for enhancing the endosomal dissociation of antigens from antigen-binding molecules and thus enhancing the FcRn binding of the antigen-binding molecules, by impairing the antigen-binding ability of an antigen-binding molecule at the endosomal pH (acidic pH) as compared to that at the plasma pH (neutral pH). When the antigen is a soluble antigen, the antigen-binding molecule can bind to FcRn in the presence or absence of the antigen. If dissociation of the antigen from the antigen-binding molecule within endosomes can be promoted by impairing the antigen-binding ability of the antigen-binding molecule at intraendosomal (acidic) pH as compared to that at plasma (neutral) pH, the FcRn binding of the antigen-binding molecule that is "free from an antigen" can be enhanced by the methods of the present invention.

[0059] Regardless of whether an antigen is membrane-bound or soluble, if an antigen-binding molecule free from an antigen can return to plasma with FcRn, the antigen-binding molecule can bind to the antigen again. By repeating this process, the antigen-binding molecule can bind to the antigen multiple times. In the present invention, "enhancing the FcRn binding of an antigen-binding molecule within a cell" does not necessarily mean that every antigen-binding molecule binds to FcRn. It is acceptable that the proportion of an antigen-binding molecule free from an antigen that binds to FcRn within a cell increases when compared to before impairing the antigen-binding ability of the antigen-binding molecule at the endosomal pH as compared to that at the plasma pH. Preferred antigen-binding molecules in the methods of the present invention for enhancing the intracellular binding between the antigen-binding molecule and FcRn include, for example, antigen-binding molecules that bind to membrane-bound antigens (membrane antigens) such as membrane proteins. Other preferable antigen-binding molecules include antigen-binding molecules that bind to soluble antigens such as soluble proteins.

[0060] The methods of enhancing the binding of an antigen-binding molecule and FcRn within a cell are alternatively expressed as the methods of promoting the FcRn binding of an antigen-binding molecule within a cell, for example, within endosomes.

[0061] Furthermore, the present invention relates to methods for releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an antigen-free form to the outside of the cell, by impairing the antigen-binding ability of the antigen-binding molecule at acidic pH as compared that at neutral pH. In the present invention, "releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an antigen-free form to the outside of the cell" does not necessarily mean that every antigen-binding molecule, which has been bound to an antigen and internalized into a cell, is released in an antigen-free form to the outside of the cell. It is acceptable that the proportion of antigen-binding molecules that are released to the outside of the cell increases when compared to before impairing the antigen-binding ability of the antigen-binding molecule at acidic pH as compared to that at neutral pH. It is preferred that the antigen-binding molecule released to the outside of a cell retains the antigen-binding ability. Furthermore, the method of releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an antigen-free form to the outside of the cell can also be referred to as a method of conferring to the antigen-binding molecule a property that the antigen-binding molecule becomes more easily released to the outside of the cell in an antigen-free form when the antigen-binding molecule is bound to an antigen and internalized into a cell.

[0062] Furthermore, the present invention relates to methods for increasing the ability of the antigen-binding molecules to eliminate antigens in plasma by impairing the antigen-binding ability of the antigen-binding molecules at acidic pH as compared to that at neutral pH. In the present invention "the ability to eliminate antigens in plasma" refers to the ability to eliminate from plasma antigens that are present in plasma, when the antigen-binding molecules are administered *in vivo* or are secreted *in vivo*. Thus, in the present invention, "increasing the ability of the antigen-binding molecule to eliminate antigen in plasma" means that the rate of elimination of antigens from plasma when the antigen-binding molecules are administered *in vivo* is accelerated as compared to that before lowering the antigen-binding ability of the antigen-binding molecules at acidic pH as compared to that at neutral pH. Whether the ability of the antigen-binding molecule to eliminate antigens in plasma is increased can be determined by, for example, administering soluble antigens

and antigen-binding molecules *in vivo*, and then measuring the concentration of soluble antigens in plasma. When the concentration of soluble antigens in plasma after the administration of soluble antigens and antigen-binding molecules is reduced by lowering the antigen-binding ability of the antigen-binding molecule at acidic pH than that at neutral pH, it can be determined that the ability of the antigen-binding molecule to eliminate antigens in plasma is increased.

**[0063]** The present invention also relates to methods for improving the pharmacokinetics of an antigen-binding molecule by substituting histidine or non-natural amino acid for at least one amino acid in the antigen-binding molecule or inserting histidine or non-natural amino acid into the molecule.

**[0064]** Furthermore, the present invention provides methods for increasing the number of times of antigen-binding of an antigen-binding molecule by substituting histidine or non-natural amino acid for at least one amino acid in the antigen-binding molecule or inserting histidine or non-natural amino acid into the molecule.

**[0065]** In addition, the present invention relates to methods for increasing the number of antigens that can be bound by an antigen-binding molecule by substituting histidine or non-natural amino acid for at least one amino acid in the antigen-binding molecule or inserting histidine or non-natural amino acid into the molecule.

**[0066]** The present invention also provides methods for dissociating an antigen within a cell from an extracellularly-bound antigen-binding molecule by substituting at least one amino acid in the antigen-binding molecule with histidine or non-natural amino acid, or inserting histidine or non-natural amino acid into the molecule.

**[0067]** The present invention also provides methods for releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an antigen-free form to the outside of the cell by substituting at least one amino acid in the antigen-binding molecule with histidine or non-natural amino acid, or inserting histidine or non-natural amino acid into the molecule.

**[0068]** The present invention also provides methods for increasing the ability of the antigen-binding molecule to eliminate antigens in plasma by substituting at least one amino acid in the antigen-binding molecule with histidines or non-natural amino acid, or inserting histidine or non-natural amino acid into the molecule.

**[0069]** The site of histidine or non-natural amino acid mutation (substitution, insertion, etc.) is not particularly limited. A histidine or non-natural amino acid may be substituted or inserted at any site. Preferred sites of histidine or non-natural amino acid substitution or insertion include, for example, sites within a region that has an impact on the antigen-binding ability of the antigen-binding molecule. For example, when the antigen-binding molecule is an antibody, such sites include an antibody variable region or CDR. The number of histidine or non-natural amino acid mutations is not particularly limited. Histidine or non-natural amino acid may be substituted or inserted at a single site, or at two or more sites. Furthermore, a deletion, addition, insertion, and/or substitution of other amino acids may be introduced simultaneously with the histidine or non-natural amino acid substitution or insertion.

**[0070]** In the present invention, when the antigen-binding molecule is an antibody, possible sites of histidine or non-natural amino acid substitution include, for example, sites within the CDR sequence or sequence responsible for the CDR structure of an antibody. Such sites include, for example, the sites listed below. The amino acid positions are numbered based on the Kabat numbering (Kabat EA *et al.*, (1991) Sequences of Proteins of Immunological Interest, NIH).

**[0071]** Heavy chain: H27, H31, H32, H33, H35, H50, H58, H59, H61, H62, H63, H64, H65, H99, H100b, and H102

**[0072]** Light chain: L24, L27, L28, L32, L53, L54, L56, L90, L92, and L94

**[0073]** Among the above sites, H32, H61, L53, L90, and L94 could be universal modification sites.

**[0074]** When the antigen is the IL-6 receptor (*e.g.*, human IL-6 receptor), preferable modification sites include the following. However, the modification sites are not particularly limited thereto.

**[0075]** Heavy chain: H27, H31, H32, H35, H50, H58, H61, H62, H63, H64, H65, H100b, and H102

**[0076]** Light chain: L24, L27, L28, L32, L53, L56, L90, L92, and L94

**[0077]** When histidine or non-natural amino acid is substituted at multiple sites, preferred combinations of substitution sites include, for example, the combination of H27, H31, and H35; combination of H27, H31, H32, H35, H58, H62, and H102; combination of L32 and L53; and combination of L28, L32, and L53. In addition, preferred combinations of substitution sites of heavy and light chains include the combination of H27, H31, L32, and L53.

**[0078]** When the antigen is IL-6 (*e.g.*, human IL-6), preferable modification sites include the following. However, the modification sites are not particularly limited thereto.

**[0079]** Heavy chain: H32, H59, H61, and H99

**[0080]** Light chain: L53, L54, L90, and L94

**[0081]** When the antigen is the IL-31 receptor (*e.g.*, human IL-31 receptor), preferable modification sites include H33. However, the modification sites are not particularly limited thereto.

**[0082]** Regarding the above sites, only one site may be substituted with histidine or non-natural amino acid. Alternatively, multiple sites may be substituted with histidine or non-natural amino acid.

**[0083]** The methods of the present invention are applicable to any antigen-binding molecules, regardless of the type of target antigen.

**[0084]** The antigen-binding molecules of the present invention are not particularly limited as long as they have the specific binding activity to an antigen of interest. Preferred antigen-binding molecules of the present invention include,



for example, substances having an antigen-binding domain of an antibody. The antigen-binding domain of an antibody includes, for example, CDR and variable region. When the antigen-binding domain of an antibody is CDR, the antigen-binding molecule may include all of the six CDRs of a whole antibody, or one, or two or more of them. Alternatively, when an antigen-binding molecule includes CDR as a binding domain of an antibody, the CDR may include amino acid

deletion, substitution, addition, and/or insertion, or may be a partial CDR.

**[0085]** Furthermore, when the antigen-binding molecule includes an antibody constant region, the present invention relates to methods for improving the pharmacokinetics of antigen-binding molecules by modification (for example, amino acid substitution, deletion, addition, and/or insertion) of the antibody constant region in the antigen-binding molecule.

**[0086]** In addition, when the antigen-binding molecule includes an antibody constant region, the present invention provides methods for increasing the number of times of antigen-binding of an antigen-binding molecule by modification (for example, amino acid substitution, deletion, addition, and/or insertion) of the antibody constant region in the antigen-binding molecule.

**[0087]** Furthermore, when the antigen-binding molecule includes an antibody constant region, the present invention relates to methods for increasing the number of antigens that can be bound by an antigen-binding molecule by modification (for example, amino acid substitution, deletion, addition, and/or insertion) of the antibody constant region in the antigen-binding molecule.

**[0088]** Furthermore, when the antigen-binding molecule includes an antibody constant region, the present invention relates to methods for dissociating within a cell an antigen from an extracellularly-bound antigen-binding molecule by modification (for example, amino acid substitution, deletion, addition, and/or insertion) of the antibody constant region in the antigen-binding molecule.

**[0089]** Furthermore, when the antigen-binding molecule includes an antibody constant region, the present invention relates to methods for releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an antigen-free form to the outside of the cell, by modification (for example, amino acid substitution, deletion, addition, and/or insertion) of the antibody constant region in the antigen-binding molecule.

**[0090]** Furthermore, when the antigen-binding molecule includes an antibody constant region, the present invention relates to methods for increasing the ability of an antigen-binding molecule to eliminate antigens in plasma by modification (for example, amino acid substitution, deletion, addition, and/or insertion) of the antibody constant region in the antigen-binding molecule.

**[0091]** In a preferred embodiment, the antigen-binding substance of the present invention includes antigen-binding substances including an FcRn-binding region. After internalized into cells, antigen-binding substances including an FcRn-binding region can return to the plasma by the FcRn salvage pathway. The FcRn-binding region is preferably a domain that directly binds to FcRn. Preferred FcRn-binding region includes, for example, antibody Fc regions.

**[0092]** However, the FcRn-binding region of the present invention may be a region that can bind to a polypeptide having the ability to bind to FcRn such as albumin or IgG, since such region that can bind to the polypeptide having FcRn-binding ability can binds indirectly to FcRn via albumin, IgG, etc.

**[0093]** Antigens recognized by antigen-binding molecules such as antibodies of interest in the methods of the present invention are not particularly limited. Such antibodies of interest may recognize any antigen. Antibodies whose pharmacokinetics is to be improved by the methods of the present invention include, for example, antibodies that recognize membrane antigens such as receptor proteins (membrane-bound receptors and soluble receptors) and cell surface markers, and antibodies that recognize soluble antigens such as cytokines. Preferred examples of membrane antigens of the present invention include membrane proteins. Examples of soluble antigens of the present invention include soluble proteins. Antigens recognized by antibodies whose pharmacokinetics is to be improved by the methods of the present invention include, for example, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-15, IL-31, IL-23, IL-2 receptor, IL-6 receptor, OSM receptor, gp130, IL-5 receptor, CD40, CD4, Fas, osteopontin, CRTH2, CD26, PDGF-D, CD20, monocyte chemotactic factor, CD23, TNF- $\alpha$ , HMGB-1,  $\alpha$ 4 integrin, ICAM-1, CCR2, CD11a, CD3, IFN $\gamma$ , BlyS, HLA-DR, TGF- $\beta$ , CD52, and IL-31 receptor. Particularly preferred antigens include IL-6 receptor.

**[0094]** Furthermore, the antigen-binding molecule of interest in the methods of the present invention includes antigen-binding molecules having an antagonistic activity (antagonistic antigen-binding molecules) and antigen-binding molecules having an agonistic activity (agonistic antigen-binding molecules). In a preferred embodiment, the antigen-binding molecule includes antagonistic antigen-binding molecules, in particular, antagonistic antigen-binding molecules that recognize membrane antigens such as receptors, or soluble antigens such as cytokines. For example, an antagonistic antigen-binding molecule that recognizes a receptor inhibits the ligand-receptor binding by binding to the receptor, and thus inhibits the signaling mediated via the receptor.

**[0095]** In the present invention, the antigen-binding molecule or interest is not particularly limited, and may be any antigen-binding molecules. The antigen-binding molecule of the present invention preferably includes both antigen-binding activity (antigen-binding region) and FcRn-binding region. In particular, preferred antigen-binding molecule of the present invention includes a region that binds to human FcRn. The antigen-binding molecule including both antigen-binding activity and FcRn-binding region includes, for example, antibodies. The antibodies preferred in the context of

the present invention include, for example, IgG antibodies. When the antibody to be used is an IgG antibody, the type of IgG is not limited; the IgG belonging to any isotype (subclass) such as IgG1, IgG2, IgG3, or IgG4 can be used. Furthermore, amino acid mutations (*e.g.*, M73) may be introduced into the constant region of any of these IgG isotypes. Amino acid mutations to be introduced include, for example, those potentiate or impair the binding to Fcγ receptor (Proc Natl Acad Sci U S A. 2006 Mar 14;103(11):4005-10) and those potentiate or impair the binding to FcRn (J Biol Chem. 2001 Mar 2;276(9):6591-604), but are not limited to these examples. Alternatively, it is also possible to alter the pH-dependent binding by selecting an appropriate constant region such as of IgG2.

**[0096]** When the antigen-binding molecule of interest of the present invention is an antibody, it may be an antibody derived from any animal, such as a mouse antibody, human antibody, rat antibody, rabbit antibody, goat antibody, or camel antibody. Furthermore, the antibody may be a modified antibody, for example, a chimeric antibody, and in particular, a modified antibody including amino acid substitution in the sequence of a humanized antibody, etc. The antibodies also include bispecific antibodies, antibody modification products linked with various molecules, and polypeptides including antibody fragments.

**[0097]** "Chimeric antibodies" are antibodies prepared by combining sequences derived from different animals. Specifically, the chimeric antibody includes, for example, antibodies having heavy and light chain variable (V) regions from a mouse antibody and heavy and light chain constant (C) regions from a human antibody.

**[0098]** "Humanized antibodies", also referred to as reshaped human antibodies, are antibodies in which complementarity determining regions (CDRs) of an antibody derived from a nonhuman mammal, for example, a mouse, are transplanted into the CDRs of a human antibody. Methods for identifying CDRs are known (Kabat et al., Sequence of Proteins of Immunological Interest (1987), National Institute of Health, Bethesda, Md.; Chothia et al., Nature (1989) 342:877). General genetic recombination technologies suitable for this purpose are also known (see European Patent Application EP 125023; and WO 96/02576).

**[0099]** Bispecific antibody refers to an antibody that has, in the same antibody molecule, variable regions that recognize different epitopes. A bispecific antibody may be an antibody that recognizes two or more different antigens, or an antibody that recognizes two or more different epitopes on a same antigen.

**[0100]** Furthermore, polypeptides including antibody fragments include, for example, Fab fragments, F(ab')<sub>2</sub> fragments, scFv (Nat Biotechnol. 2005 Sep;23(9):1126-36), domain antibodies (dAb) (WO 2004/058821, WO 2003/002609), scFv-Fc (WO 2005/037989), dAb-Fc, and Fc fusion proteins. Of these, molecules including an Fc domain have the activity of binding to FcRn, and are therefore suitable for use in the methods discovered in the present invention.

**[0101]** Further, the antigen-binding molecules that are applicable to the present invention may be antibody-like molecules. An antibody-like molecule is a molecule that can exhibit functions by binding to a target molecule (Current Opinion in Biotechnology 2006, 17:653-658; Current Opinion in Biotechnology 2007, 18:1-10; Current Opinion in Structural Biology 1997, 7:463-469; Protein Science 2006, 15:14-27), and includes, for example, DARPin (WO 2002/020565), Affibody (WO 1995/001937), Avimer (WO 2004/044011; WO 2005/040229), and Adnectin (WO 2002/032925). If these antibody-like molecules can bind to target molecules in a pH-dependent manner, it is possible for a single molecule to bind multiple target molecules.

**[0102]** Furthermore, the antigen-binding molecule may be a receptor protein or a receptor-Fc fusion protein that binds to a target, including, for example, TNFR-Fc fusion protein, IL1R-Fc fusion protein, VEGFR-Fc fusion protein, and CTLA4-Fc fusion protein (Nat Med. 2003 Jan;9(1):47-52; BioDrugs. 2006;20(3):151-60). If such receptor proteins and receptor-Fc fusion proteins can bind to target molecules in a pH-dependent manner, it is possible for a single molecule to bind multiple target molecules.

**[0103]** Moreover, the antigen-binding molecule may be an artificial ligand protein or artificial ligand fusion protein that binds to a target and has the neutralizing effect, and includes, for example, mutant IL-6 (EMBO J. 1994 Dec 15;13(24):5863-70). If such artificial ligand proteins and artificial ligand fusion proteins can bind to target molecules in a pH-dependent manner, it is possible for a single molecule to bind multiple target molecules.

**[0104]** Furthermore, the antibodies of the present invention may include modified sugar chains. Antibodies with modified sugar chains include, for example, antibodies with modified glycosylation (WO 99/54342), antibodies that are deficient in fucose that is added to the sugar chain (WO 00/61739; WO 02/31140; WO 2006/067847; WO 2006/067913), and antibodies having sugar chains with bisecting GlcNAc (WO 02/79255).

**[0105]** Although the methods of the present invention are not limited to any specific theory, the relationship between making the antigen-binding ability at acidic pH weaker as compared to that at neutral pH, the improvement of the pharmacokinetics, and the multiple-time binding to the antigen can be explained as follows, for instance.

**[0106]** For example, when the antibody is an antibody that binds to a membrane antigen, the antibody administered into the body binds to the antigen and then is taken up via internalization into endosomes in the cells together with the antigen and while the antibody is kept bound to the antigen. Then, the antibody translocates to lysosomes while the antibody is kept bound to the antigen, and the antibody is degraded by the lysosome together with the antigen. The internalization-mediated elimination from the plasma is called antigen-dependent elimination, and such elimination has been reported with numerous antibody molecules (Drug Discov Today. 2006 Jan;11(1-2):81-8). When a single molecule

of IgG antibody binds to antigens in a divalent manner, the single antibody molecule is internalized while the antibody is kept bound to the two antigen molecules, and degraded in the lysosome. Accordingly, in the case of typical antibodies, one molecule of IgG antibody cannot bind to three or more molecules of antigen. For example, a single IgG antibody molecule having a neutralizing activity cannot neutralize three or more antigen molecules.

**[0107]** The relatively prolonged retention (slow elimination) of IgG molecules in the plasma is due to the function of FcRn which is known as a salvage receptor of IgG molecules. When taken up into endosomes via pinocytosis, IgG molecules bind to FcRn expressed in the endosomes under the acidic condition in the endosomes. While IgG molecules that did not bind to FcRn transfer to lysosomes where they are degraded, IgG molecules that bound to FcRn translocate to the cell surface and return again in the plasma by dissociating from FcRn under the neutral condition in the plasma.

**[0108]** Alternatively, when the antigen is an antigen that binds to a soluble antigen, the antibody administered into the body binds to the antigen and then is taken up into cells while the antibody is kept bound to the antigen. Many antibodies taken up into cells are released to the outside of cells via FcRn. However, since the antibodies are released to the outside of cells, with the antibodies kept bound to antigens, the antibodies cannot bind to antigens again. Thus, similar to antibodies that bind to membrane antigens, in the case of typical antibodies, one molecule of IgG antibody cannot bind to three or more antigen molecules.

**[0109]** The present inventors reasoned that, when antibodies that bound to antigens such as membrane antigens are taken up into endosomes by internalization, while the antibodies that are kept bound to the antigens translocate to lysosomes and are degraded, the IgG antibodies whose antigens dissociated in the endosomes could bind to FcRn that are expressed in the endosomes. Specifically, the present inventors discovered that an antibody that strongly binds to an antigen in the plasma but weakly binds to the antigen within the endosome can bind to an antigen in plasma and be taken up while kept forming a complex with the antigen into endosomes in the cells via internalization; dissociate from the antigen in the endosome; then bind to FcRn and translocate to the cell surface; and return again in the plasma in a state not bound to antigens to neutralize multiple membrane-bound antigens. Furthermore, the present inventors discovered that an antibody having the property of strongly binding to antigens in the plasma but weakly binding to antigens in the endosome can dissociate from the antigens in the endosome even when the antibody had bound to antigens such as soluble antigens; therefore, they are released again into the plasma in a state not bound to antigens and can neutralize multiple soluble antigens.

**[0110]** In particular, the present inventors noted that the pH in the plasma was different from the pH in the endosomes, and thus discovered that antibodies that strongly bind to antigens under plasma pH condition but that weakly bind to antigens under endosomal pH condition were superior in retention in the plasma, because one antibody molecule could bind to multiple antigens.

**[0111]** The endosomes, which are membrane vesicles, form networks in the cytoplasm of eukaryotic cells and are responsible for the metabolism of macromolecules in the process from the cell membrane to the lysosomes. The pH in the endosomes has been reported be generally an acidic pH of 5.5 to 6.0 (Nat Rev Mol Cell Biol. 2004 Feb;5(2):121-32). Meanwhile, the pH in the plasma is known to be almost neutral (normally, pH 7.4).

**[0112]** Accordingly, an antigen-binding molecule whose antigen-binding activity at acidic pH is weaker than the antigen-binding activity at neutral pH binds to the antigen in the plasma which have a neutral pH, is taken up into cells, and then dissociates from the antigen in the endosomes which have an acidic pH. The antigen-binding molecule that dissociated from the antigen binds to FcRn, translocates to the cell surface, and returns again in the plasma in a state not bound to antigens. As a result, the antigen-binding molecule can bind to antigens multiple times, and the pharmacokinetics is improved.

#### <Antigen-binding molecule substances>

**[0113]** Furthermore, the present invention provides antigen-binding molecules whose antigen-binding activity at pH 4.0 to pH 6.5 is lower than that at pH 6.7 to pH 10.0, preferably antigen-binding molecules whose antigen-binding activity at pH 5.0 to pH 6.0 is lower than that at pH 7.0 to 8.0. Specifically, antigen-binding molecules whose antigen-binding activity at pH 4.0 to pH 6.5 is lower than that at pH 6.7 to pH 10.0 include, for example, antigen-binding molecules whose antigen-binding activity at pH 5.8 is lower than that at pH 7.4. Antigen-binding molecules whose antigen-binding activity at pH 5.8 is lower than that at pH 7.4 can also be expressed as antigen-binding molecules whose antigen-binding activity at pH 7.4 is higher than that at pH 5.8.

**[0114]** As for the antigen-binding molecules of the present invention whose antigen-binding activity at pH 5.8 is lower than that at pH 7.4, so long as the antigen-binding activity at pH 5.8 is lower than the binding at pH 7.4, there is no limitation on the difference in binding activity, and the antigen-binding activity at pH 5.8 only need to be lower, even slightly.

**[0115]** A preferred embodiment of an antigen-binding molecule of the present invention whose antigen-binding activity at pH 5.8 is lower than that at pH 7.4 includes antigen-binding molecules whose antigen-binding activity at pH 7.4 is twice or greater than that at pH 5.8. A more preferred embodiment of the antigen-binding molecule includes antigen-binding molecules whose antigen-binding activity at pH 7.4 is ten times or greater than that at pH 5.8. A still more



preferred embodiment of the antigen-binding molecule includes antigen-binding molecules whose antigen-binding activity at pH 7.4 is 40 times or greater than that at pH 5.8.

[0116] Specifically, in a preferred embodiment, the antigen-binding molecule of the present invention has antigen-binding activity at pH 5.8 that is lower than that at pH 7.4, wherein the value of  $D(\text{pH}5.8)/KD(\text{pH}7.4)$ , which is a ratio of KD for the antigen at pH 5.8 and that at pH 7.4, is preferably 2 or greater, more preferably 10 or greater, and still more preferably 40 or greater. The upper limit of the  $KD(\text{pH}5.8)/KD(\text{pH}7.4)$  value is not particularly limited, and may be any value, for example, 400, 1000, or 10000, as long as production is possible using the technologies of those skilled in the art.

[0117] In another preferred embodiment, the antigen-binding molecule of the present invention whose antigen-binding activity at pH 5.8 is lower than that at pH 7.4, has a value of  $k_d(\text{pH}5.8)/k_d(\text{pH}7.4)$ , which is a ratio of the  $k_d$  for the antigen at pH 5.8 and the  $k_d$  for the antigen at pH 7.4, that is 2 or greater, more preferably 5 or greater, even more preferably 10 or greater, and still more preferably 30 or greater. The upper limit of the  $k_d(\text{pH}5.8)/k_d(\text{pH}7.4)$  value is not particularly limited, and may be any value, for example, 50, 100, or 200, as long as production is possible using the technologies of those skilled in the art.

[0118] Conditions other than the pH at which the antigen-binding activity is measured can be appropriately selected by those skilled in the art, and the conditions are not particularly limited; however, the measurements can be carried out, for example, under conditions of MES buffer and 37°C, as described in the Examples. Furthermore, the antigen-binding activity of an antigen-binding molecule can be determined by methods known to those skilled in the art, for example, using Biacore T100 (GE Healthcare) or the like, as described in the Examples.

[0119] It is presumed that such an antigen-binding molecule, which weakly binds to an antigen at acidic pH, easily dissociates from the antigen under the endosomal acidic condition, and that after internalization into cells, it binds to FcRn and is easily released to the outside of the cells. The antigen-binding molecule released to the outside of the cells without being degraded inside the cells can bind again to other antigens. Accordingly, when the antigen-binding molecule is, for example, an antigen-binding neutralizing molecule, the antigen-binding molecule that easily dissociates from the antigen under the endosomal acidic condition can bind and neutralize antigens multiple times. As a result, antigen-binding molecules whose antigen-binding activity at pH 4.0 to pH 6.5 is lower than that at pH 6.7 to pH 10.0 are superior in retention in the plasma.

[0120] In a preferred embodiment, the antigen-binding molecule whose antigen-binding activity at pH 5.8 is lower than that at pH 7.4 includes antigen-binding molecules in which at least one amino acid in the antigen-binding molecule is substituted with histidine or a non-natural amino acid, or in which at least one histidine or a non-natural amino acid has been inserted. The site into which the histidine or non-natural amino acid mutation is introduced is not particularly limited and may be any site, as long as the antigen-binding activity at pH 5.8 is weaker than that at pH 7.4 (the  $KD(\text{pH}5.8)/KD(\text{pH}7.4)$  value is greater or the  $k_d(\text{pH}5.8)/k_d(\text{pH}7.4)$  value is greater) as compared to before substitution. Examples include variable regions and CDRs of an antibody in the case the antigen-binding molecule is an antibody. The number of amino acids to be substituted with histidine or non-natural amino acid and the number of amino acids to be inserted can be appropriately determined by those skilled in the art. One amino acid may be substituted with histidine or non-natural amino acid, or one amino acid may be inserted, or two or more amino acids may be substituted with histidine or non-natural amino acids, or two or more amino acids may be inserted. Moreover, apart from the substitutions to histidine or to non-natural amino acid or insertion of histidine or of non-natural amino acid, deletion, addition, insertion, and/or substitution and such of other amino acids may also be simultaneously carried out. Substitutions to histidine or to non-natural amino acid or insertion of histidine or of non-natural amino acid may be carried out at random using a method such as histidine scanning, which uses histidine instead of alanine in alanine scanning which is known to those skilled in the art. Antigen-binding molecules whose  $KD(\text{pH}5.8)/KD(\text{pH}7.4)$  or  $k_d(\text{pH}5.8)/k_d(\text{pH}7.4)$  is increased as compared to before mutation can be selected from antigen-binding molecules into which histidine or non-natural amino acid mutation has been introduced at random.

[0121] Preferred antigen-binding molecules with mutation to histidine or to non-natural amino acid and whose antigen-binding activity at pH 5.8 is lower than that at pH 7.4 include, for example, antigen-binding molecules whose antigen-binding activity at pH 7.4 after the mutation to histidine or to non-natural amino acid is equivalent to the antigen-binding activity at pH 7.4 before the mutation to histidine or to non-natural amino acid. In the present invention, "an antigen-binding molecule after histidine or non-natural amino acid mutation has an antigen-binding activity that is equivalent to that of the antigen-binding molecule before histidine or non-natural amino acid mutation" means that, when the antigen-binding activity of an antigen-binding molecule before histidine or non-natural amino acid mutation is set as 100%, the antigen-binding activity of the antigen-binding molecule after histidine or non-natural amino acid mutation is at least 10% or more, preferably 50% or more, more preferably 80% or more, and still more preferably 90% or more. The antigen-binding activity at pH 7.4 after histidine or non-natural amino acid mutation may be greater than the antigen-binding activity at pH 7.4 before histidine or non-natural amino acid mutation. When the antigen-binding activity of the antigen-binding molecule is decreased due to substitution or insertion of histidine or non-natural amino acid, the antigen-binding activity may be adjusted by introducing substitution, deletion, addition, and/or insertion and such of one or more amino acids into the antigen-binding molecule so that the antigen-binding activity becomes equivalent to that before histidine

substitution or insertion. The present invention also includes such antigen-binding molecules whose binding activity has been made equivalent as a result of substitution, deletion, addition, and/or insertion of one or more amino acids after histidine substitution or insertion.

**[0122]** Further, when the antigen-binding molecule is a substance including an antibody constant region, in another preferred embodiment of the antigen-binding molecule whose antigen-binding activity at pH 5.8 is lower than that at pH 7.4, the present invention includes methods for modifying antibody constant regions contained in the antigen-binding molecules. Specific examples of antibody constant regions after modification include the constant regions described in the Examples.

**[0123]** When the antigen-binding activity of the antigen-binding substance at pH 5.8 is weakened compared to that at pH 7.4 (when  $KD(pH5.8)/KD(pH7.4)$  value is increased) by the above described methods and such, it is generally preferable that the  $KD(pH5.8)/KD(pH7.4)$  value is two times or more, more preferably five times or more, and even more preferably ten times or more as compared to that of the original antibody, but is not particularly limited thereto.

**[0124]** The antigen-binding molecules of the present invention may further have any other property, as long as their antigen-binding activity at pH 4.0 to pH 6.5 is lower than that at pH 6.7 to pH 10.0. For example, the antigen-binding molecules may be agonistic or antagonistic antigen-binding molecules. Preferred antigen-binding molecules of the present invention include, for example, antagonistic antigen-binding molecules. In general, an antagonistic antigen-binding molecule inhibits receptor-mediated intracellular signaling by inhibiting the binding between a ligand (agonist) and the receptor.

**[0125]** Furthermore, the present invention provides antibodies in which amino acid on at least one site indicated below is substituted with histidine or non-natural amino acid. Amino acid positions are indicated based on the Kabat numbering (Kabat EA et al., (1991) Sequences of Proteins of Immunological Interest, NIH).

**[0126]** Heavy chain: H27, H31, H32, H33, H35, H50, H58, H59, H61, H62, H63, H64, H65, H99, H100b, and H102

**[0127]** Light chain: L24, L27, L28, L32, L53, L54, L56, L90, L92, and L94

**[0128]** Among the above sites, H32, H61, L53, L90, and L94 could be universal modification sites.

**[0129]** When the antigen is the IL-6 receptor (*e.g.*, human IL-6 preceptor), preferable modification sites include the following. However, the modification sites are not particularly limited thereto.

**[0130]** Heavy chain: H27, H31, H32, H35, H50, H58, H61, H62, H63, H64, H65, H100b, and H102

**[0131]** Light chain: L24, L27, L28, L32, L53, L56, L90, L92, and L94

**[0132]** When histidine or non-natural amino acid is substituted at multiple sites, preferred combinations of substitution sites include, for example, the combination of H27, H31, and H35; combination of H27, H31, H32, H35, H58, H62, and H102; combination of L32 and L53; and combination of L28, L32, and L53. In addition, preferred combinations of substitution sites of heavy and light chains include the combination of H27, H31, L32, and L53.

**[0133]** When the antigen is IL-6 (*e.g.*, human IL-6), preferable modification sites include the following. However, the modification sites are not particularly limited thereto.

**[0134]** Heavy chain: H32, H59, H61, and H99

**[0135]** Light chain: L53, L54, L90, and L94

**[0136]** When the antigen is the IL-31 receptor (*e.g.*, human IL-31 receptor), preferable modification sites include H33. However, the modification sites are not particularly limited thereto.

**[0137]** The antigen-binding molecules of the present invention may recognize any antigen. Antigens recognized by antibodies of the present invention specifically include the above-mentioned receptor proteins (membrane-bound receptors or soluble receptors), membrane antigens such as cell surface markers, and soluble antigens such as cytokines, for example, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-15, IL-31, IL-23, IL-2 receptor, IL-6 receptor, OSM receptor, gp130, IL-5 receptor, CDR40, CD4, Fas, osteopontin, CRTH2, CD26, PDGF-D, CD20, monocyte chemoattractant factor, CD23, TNF- $\alpha$ , HMGB-1,  $\alpha$ 4 integrin, ICAM-1, CCR2, CD11a, CD3, IFN $\gamma$ , BLYS, HLA-DR, TGF- $\beta$ , CD52, and IL-31 receptor.

**[0138]** Particularly preferred antigens include the IL-6 receptor.

**[0139]** The antigen-binding molecules of the present invention are described above.

**[0140]** In a preferred embodiment of the present invention, the antigen-binding molecules include antibodies. Antibodies having antigen-binding activity and FcRn-binding region include, for example, IgG antibodies. When the antibody used is an IgG antibody, there is no limitation as to its type. It is possible to use IgG1, IgG2, IgG3, IgG4, and such.

**[0141]** The origin of antibody of the present invention is not particularly limited, and may be of any origin. It is possible to use, for example, mouse antibodies, human antibodies, rat antibodies, rabbit antibodies, goat antibodies, camel antibodies, and others. Furthermore, the antibodies may be, for example, the above-described chimeric antibodies, and in particular, modified antibodies with amino acid sequence substitutions, such as humanized antibodies. The antibodies may also be the above-described bispecific antibodies, antibody modification products to which various molecules have been linked, polypeptides including antibody fragments, and antibodies with modified sugar chains.

**[0142]** Generation of chimeric antibodies is known. In the case of a human-mouse chimeric antibody, for example, a DNA encoding an antibody V region may be linked to a DNA encoding a human antibody C region; this can be inserted

into an expression vector and introduced into a host to produce the chimeric antibody.

**[0143]** "Humanized antibodies" are also referred to as reshaped human antibodies, and are antibodies in which the complementarity determining region (CDR) of a nonhuman mammal, for example a mouse, is transplanted to the CDR of a human antibody. Methods for identifying CDRs are known (Kabat et al., Sequence of Proteins of Immunological Interest (1987), National Institute of Health, Bethesda, Md.; Chothia et al., Nature (1989) 342:877). General genetic recombination technologies suitable for this purpose are also known (see European Patent Application EP 125023; and WO 96/02576). Humanized antibodies can be produced by known methods, for example, the CDR of a mouse antibody can be determined, and a DNA encoding an antibody in which the CDR is linked to the framework region (FR) of a human antibody is obtained. Humanized antibodies can then be produced using a system that uses conventional expression vectors. Such DNAs can be synthesized by PCR, using as primers several oligonucleotides prepared to have portions that overlap with the end regions of both the CDR and FR (see the method described in WO 98/13388). Human antibody FRs linked via CDRs are selected such that the CDRs form a suitable antigen binding site. If required, amino acids in the FRs of an antibody variable region may be substituted so that the CDRs of the reshaped human antibody can form a suitable antigen binding site (Sato, K. et al., Cancer Res. (1993) 53:10.01-6). Amino acid residues in the FRs that can be modified include portions that directly bind to an antigen via non-covalent bonds (Amit et al., Science (1986) 233: 747-53), portions that influence or have an effect on the CDR structure (Chothia et al., J. Mol. Biol. (1987) 196: 901-17), and portions involved in VH-VL interactions (EP 239400).

**[0144]** When the antibodies of the present invention are chimeric antibodies or humanized antibodies, the C regions of these antibodies are preferably derived from human antibodies. For example, C $\gamma$ 1, C $\gamma$ 2, C $\gamma$ 3, and C $\gamma$ 4 can be used for the H chain, while C $\kappa$  and C $\lambda$  can be used for the L chain. Moreover, if required, amino acid mutations may be introduced into the human antibody C region to enhance or lower the binding to Fc $\gamma$  receptor or FcRn or to improve antibody stability or productivity. A chimeric antibody of the present invention preferably includes a variable region of an antibody derived from a nonhuman mammal and a constant region derived from a human antibody. Meanwhile, a humanized antibody preferably includes CDRs of an antibody derived from a nonhuman mammal and FRs and C regions derived from a human antibody. The constant regions derived from human antibodies preferably include an FcRn-binding region. Such antibodies include, for example, IgGs (IgG1, IgG2, IgG3, and IgG4). The constant regions used for the humanized antibodies of the present invention may be constant regions of antibodies of any isotype. A constant region of human IgG is preferably used, though it is not limited thereto. The FRs derived from a human antibody, which are used for the humanized antibodies, are not particularly limited either, and may be derived from an antibody of any isotype.

**[0145]** The variable and constant regions of chimeric and humanized antibodies of the present invention may be modified by deletion, substitution, insertion, and/or addition, and such, so long as the binding specificity of the original antibodies is exhibited.

**[0146]** Since the immunogenicity in the human body is lowered, chimeric and humanized antibodies using human-derived sequences are thought to be useful when administered to humans for therapeutic purposes or such.

**[0147]** The antibodies of the present invention may be prepared by any method. For example, antibodies whose antigen-binding activity at pH 5.8 is originally greater than or comparable to that at pH 7.4 may be artificially modified through histidine substitution described above or the like so that their antigen-binding activity at pH 5.8 becomes lower than that at pH 7.4. Alternatively, antibodies whose antigen-binding activity at pH 5.8 is lower than that at pH 7.4 may be selected by screening a number of antibodies obtained from an antibody library or hybridomas as described below.

**[0148]** When histidine is substituted for amino acids in an antibody, known sequences may be used for the H chain or L chain amino acid sequence of the antibody before introduction of histidine mutations, or amino acid sequences of antibodies newly obtained by methods known to those skilled in the art can also be used. For example, the antibodies may be obtained from an antibody library, or they may be obtained by cloning genes encoding antibodies from hybridomas producing monoclonal antibodies.

**[0149]** Regarding antibody libraries, many antibody libraries are already known, and methods for producing antibody libraries are also known; therefore, those skilled in the art can appropriately obtain antibody libraries. For example, regarding antibody phage libraries, one can refer to the literature such as Clackson et al., Nature 1991, 352: 624-8; Marks et al., J. Mol. Biol. 1991, 222: 581-97; Waterhouses et al., Nucleic Acids Res. 1993, 21: 2265-6; Griffiths et al., EMBO J. 1994, 13: 324.0-60; Vaughan et al., Nature Biotechnology 1996, 14: 309-14; and Japanese Patent Kohyo Publication No. (JP-A) H20-504970 (unexamined Japanese national phase publication corresponding to a non-Japanese international publication). In addition, it is possible to use known methods, such as methods using eukaryotic cells as libraries (WO 95/15393) and ribosome display methods. Furthermore, technologies to obtain human antibodies by panning using human antibody libraries are also known. For example, variable regions of human antibodies can be expressed on the surface of phages as single chain antibodies (scFvs) using phage display methods, and phages that bind to antigens can be selected. Genetic analysis of the selected phages can determine the DNA sequences encoding the variable regions of human antibodies that bind to the antigens. One the DNA sequences of scFvs that bind to the antigens is revealed, suitable expression vectors can be produced based on these sequences to obtain human antibodies. These methods are already well known, and one can refer to WO 92/01047, WO 92/20791, WO 93/06213, WO 93/11236,

WO 93/19172, WO 95/01438, and WO 95/15388.

**[0150]** As for methods for obtaining genes encoding antibodies from hybridomas, known technologies may be basically used, which involve the use of desired antigens or cells expressing the desired antigens as sensitizing antigens, using these to perform immunizations according to conventional immunization methods, fusing the resulting immune cells with known parent cells by conventional cell fusion methods, screening monoclonal antibody producing cells (hybridomas) by conventional screening methods, synthesizing cDNAs of antibody variable regions (V regions) from mRNAs of the obtained hybridomas using reverse transcriptase, and linking them with DNAs encoding the desired antibody constant regions (C regions).

**[0151]** More specifically, sensitizing antigens to obtain the above-described antibody genes encoding the H chains and L chains include both complete antigens with immunogenicity and incomplete antigens including haptens and the like with no antigenicity; however they are not limited to these examples. For example, it is possible to use whole proteins and partial peptides of proteins of interest. In addition, it is known that substances comprising polysaccharides, nucleic acids, lipids, and such can be antigens. Thus, the antigens of the antibodies of the present invention are not particularly limited. The antigens can be prepared by methods known to those skilled in the art, for example, by baculovirus-based methods (for example, WO 98/46777) and such. Hybridomas can be produced, for example, by the method of Milstein *et al.* (G. Kohler and C. Milstein, *Methods Enzymol.* 1981, 73: 3-46) and such. When the immunogenicity of an antigen is low, immunization may be performed after linking the antigen with a macromolecule having immunogenicity, such as albumin. Alternatively, if necessary, antigens may be converted into soluble antigens by linking them with other molecules. When transmembrane molecules such as membrane antigens (for example, receptors) are used as antigens, portions of the extracellular regions of the membrane antigens can be used as a fragment, or cells expressing transmembrane molecules on their cell surface may be used as immunogens.

**[0152]** Antibody-producing cells can be obtained by immunizing animals using appropriate sensitizing antigens described above. Alternatively, antibody-producing cells can be prepared by *in vitro* immunization of lymphocytes that can produce antibodies. Various mammals can be used for immunization; such commonly used animals include rodents, lagomorphs, and primates. Such animals include, for example, rodents such as mice, rats, and hamsters; lagomorphs such as rabbits; and primates including monkeys such as cynomolgus monkeys, rhesus monkeys, baboons, and chimpanzees. In addition, transgenic animals carrying human antibody gene repertoires are also known, and human antibodies can be obtained by using these animals (see WO 96/34096; Mendez *et al.*, *Nat. Genet.* 1997, 15: 146-56). Instead of using such transgenic animals, for example, desired human antibodies having binding activity against antigens can be obtained by *in vitro* sensitization of human lymphocytes with desired antigens or cells expressing the desired antigens, and then fusing the sensitized lymphocytes with human myeloma cells such as U266 (see Japanese Patent Application Kokoku Publication No. (JP-B) H01-59878 (examined, approved Japanese patent application published for opposition)). Furthermore, desired human antibodies can be obtained by immunizing transgenic animals carrying a complete repertoire of human antibody genes, with desired antigens (see WO 93/12227, WO 92/03918, WO 94/02602, WO 96/34096, and WO 96/33735).

**[0153]** Animal immunization can be carried out by appropriately diluting and suspending a sensitizing antigen in phosphate buffered saline (PBS), physiological saline, or such, and mixing it with an adjuvant to emulsify, if necessary. This is then intraperitoneally or subcutaneously injected into animals. Then, the sensitizing antigen mixed with Freund's incomplete adjuvant is preferably administered several times every four to 21 days. Antibody production can be confirmed by measuring the titer of the antibody of interest in animal sera using conventional methods.

**[0154]** Antibody-producing cells obtained from lymphocytes or animals immunized with a desired antigen can be fused with myeloma cells to generate hybridomas using conventional fusing agents (for example, polyethylene glycol) (Goding, *Monoclonal Antibodies: Principles and Practice*, Academic Press, 1986, 59-103). When required, hybridoma cells can be cultured and grown, and the binding specificity of the antibody produced from these hybridomas can be measured using known analysis methods, such as immunoprecipitation, radioimmunoassay (RIA), and enzyme-linked immunosorbent assay (ELISA). Thereafter, hybridomas producing antibodies of interest whose specificity, affinity, or activity has been determined can be subcloned by methods such as limiting dilution.

**[0155]** Next, genes encoding the selected antibodies can be cloned from hybridomas or antibody-producing cells (sensitized lymphocytes, and such) using probes that can specifically bind to the antibodies (for example, oligonucleotides complementary to sequences encoding the antibody constant regions). It is also possible to clone the genes from mRNA using RT-PCR. Immunoglobulins are classified into five different classes, IgA, IgD, IgE, IgG, and IgM. These classes are further divided into several subclasses (isotypes) (for example, IgG-1, IgG-2, IgG-3, and IgG-4; IgA-1 and IgA-2; and such). H chains and L chains used in the present invention to produce antibodies are not particularly limited and may originate from antibodies belonging to any of these classes or subclasses; however, IgG is particularly preferred.

**[0156]** Herein, it is possible to modify H-chain-encoding genes and L-chain-encoding genes using genetic engineering technologies. Genetically modified antibodies, such as chimeric antibodies and humanized antibodies, which have been artificially modified for the purpose of decreasing heterologous immunogenicity and such against humans, can be appropriately produced for antibodies such as mouse antibodies, rat antibodies, rabbit antibodies, hamster antibodies,



sheep antibodies, and camel antibodies. Chimeric antibodies are antibodies including H chain and L chain variable regions of nonhuman mammal antibody, such as mouse antibody, and the H chain and L chain constant regions of human antibody. Chimeric antibodies can be obtained by ligating a DNA encoding a variable region of a mouse antibody to a DNA encoding a constant region of a human antibody, inserting this into an expression vector, and introducing the vector into a host to produce the antibody. A humanized antibody, which is also called a reshaped human antibody, can be synthesized by PCR using several oligonucleotides produced so that they have overlapping portions at the ends of DNA sequences designed to link the complementarity determining regions (CDRs) of an antibody of a nonhuman mammal such as a mouse. The resulting DNA can be ligated to a DNA encoding a human antibody constant region. The ligated DNA can be inserted into an expression vector, and the vector can be introduced into a host to produce the antibody (see EP 239400 and WO 96/02576). Human antibody FRs that are ligated via the CDR are selected when the CDR forms a favorable antigen-binding site. If necessary, amino acids in the framework region of an antibody variable region may be substituted such that the CDR of the reshaped human antibody forms an appropriate antigen-binding site (K. Sato et al., Cancer Res. 1993, 53: 10.01-10.06).

**[0157]** In addition to the humanization described above, antibodies may be modified to improve their biological properties, for example, the binding to the antigen. In the present invention, such modifications can be achieved by methods such as site-directed mutagenesis (see for example, Kunkel (1910.0) Proc. Natl. Acad. Sci. USA 82: 488), PCR mutagenesis, and cassette mutagenesis. In general, mutant antibodies whose biological properties have been improved show amino acid sequence homology and/or similarity of 70% or higher, more preferably 80% or higher, and even more preferably 90% or higher (for example, 95% or higher, 97%, 98%, or 99%), when compared to the amino acid sequence of the original antibody variable region. Herein, sequence homology and/or similarity is defined as the ratio of amino acid residues that are homologous (same residue) or similar (amino acid residues classified into the same group based on the general properties of amino acid side chains) to the original antibody residues, after the sequence homology value has been maximized by sequence alignment and gap introduction, if necessary. In general, natural amino acid residues are classified into groups based on the characteristics of their side chains as follows:

- (1) hydrophobic: alanine, isoleucine, valine, methionine, and leucine;
- (2) neutral hydrophilic: asparagine, glutamine, cysteine, threonine, and serine;
- (3) acidic: aspartic acid and glutamic acid;
- (4) basic: arginine, histidine, and lysine;
- (5) residues that affect the orientation of the chain: glycine, and proline; and
- (6) aromatic: tyrosine, tryptophan, and phenylalanine.

**[0158]** In general, a total of six complementarity determining regions (CDRs; hypervariable regions) present on the H chain and L chain variable regions interact with each other to form an antigen-binding site of an antibody. A variable region alone is also known to be capable of recognizing and binding to an antigen, although its affinity is lower than the affinity of the whole binding site. Thus, antibody genes encoding the H chain and L chain of the present invention may encode fragments each including the H chain or L chain antigen-binding site, as long as the polypeptide encoded by the gene retains the activity of binding to the desired antigen.

**[0159]** As described above, the heavy chain variable region is in general constituted by three CDRs and four FRs. In a preferred embodiment of the present invention, amino acid residues to be "modified" can be appropriately selected from amino acid residues, for example, in a CDR or FR. In general, modifications of amino acid residues in the CDRs may reduce the antigen-binding ability. Thus, appropriate amino acid residues to be "modified" in the present invention are preferably selected from amino acid residues in the FRs, but are not limited thereto. It is possible to select amino acids in a CDR as long as the modification has been confirmed not to reduce the binding ability. Alternatively; by using public databases or such, those skilled in the art can obtain appropriate sequences that can be used as an FR of antibody variable region of an organism such as human or mouse.

**[0160]** Furthermore, the present invention provides genes encoding the antibodies of the present invention. The genes encoding the antibodies of the present invention may be any genes, and may be DNAs, RNAs, nucleic acid analogs, or the like.

**[0161]** Furthermore, the present invention also provides host cells carrying the genes described above. The host cells are not particularly limited and include, for example, *E. coli* and various animal cells. The host cells may be used, for example, as a production system to produce and express the antibodies of the present invention. *In vitro* and *in vivo* production systems are available for polypeptide production systems. Such *in vitro* production systems include, for example, production systems using eukaryotic cells or prokaryotic cells.

**[0162]** Eukaryotic cells that can be used as host cells include, for example, animal cells, plant cells, and fungal cells. Animal cells include: mammalian cells, for example, CHO (J. Exp. Med. (1995) 108: 94.0), COS, HEK293, 3T3, myeloma, BHK (baby hamster kidney), HeLa, and Vero; amphibian cells such as *Xenopus laevis* oocytes (Valle et al., Nature (1981) 291: 338-340); and insect cells such as Sf9, Sf21, and Tn5. CHO-DG44, CHO-DX11B, COS7 cells, HEK293

cells, and BHK cells are preferably used to express the antibodies of the present invention. Among animal cells, CHO cells are particularly preferable for large-scale expression. Vectors can be introduced into host cells, for example, by calcium phosphate methods, DEAE-dextran methods, methods using cationic liposome DOTAP (Boehringer-Mannheim), electroporation methods, and lipofection methods.

**[0163]** Regarding plant cells, for example, *Nicotiana tabacum*-derived cells and duckweed (*Lemna minor*) are known as a protein production system. Calluses can be cultured from these cells to produce the antibodies of the present invention. Regarding fungal cells, known protein expression systems are those using yeast cells, for example, cells of genus *Saccharomyces* (such as *Saccharomyces cerevisiae* and *Saccharomyces pombe*); and cells of filamentous fungi, for example, genus *Aspergillus* (such as *Aspergillus niger*). These cells can be used as a host to produce the antibodies of the present invention.

**[0164]** Bacterial cells can be used in the prokaryotic production systems. Regarding bacterial cells, production systems using *Bacillus subtilis* are known in addition to the production systems using *E. coli* described above. Such systems can be used in producing the antibodies of the present invention.

#### <Screening methods>

**[0165]** The present invention provides methods of screening for antigen-binding molecules whose antigen-binding activity at acidic pH is lower than that at neutral pH. The present invention also provides methods of screening for antigen-binding molecules which can individually bind to multiple antigens. The present invention also provides methods of screening for antigen-binding molecules which are superior in the retention in plasma. The present invention also provides methods of screening for an antigen-binding molecule that dissociates within a cell from an extracellularly-bound antigen. The present invention also provides methods of screening for an antigen-binding molecule that is bound to an antigen and internalized into a cell, and released to the outside of the cell in an antigen-free form. The present invention also provides methods of screening for an antigen-binding molecule that has increased ability to eliminate antigens in plasma. Furthermore, the present invention also provides methods of screening for antigen-binding molecules which are particularly useful when used as pharmaceutical compositions.

**[0166]** Specifically, the present invention provides methods of screening for antigen-binding molecules, which comprise the steps of:

- (a) determining the antigen-binding activity of an antigen-binding molecule at pH 6.7 to pH 10.0;
- (b) determining the antigen-binding activity of the antigen-binding molecule at pH 4.0 to pH 6.5; and
- (c) selecting an antigen-binding molecule whose antigen-binding activity at pH 6.7 to pH 10.0 is greater than the antigen-binding activity at pH 4.0 to pH 6.5.

**[0167]** In the screening methods of the present invention, the antigen-binding activity of the antigen-binding molecule at pH 6.7 to pH 10.0 is not particularly limited, as long as it is an antigen-binding activity at a pH between pH 6.7 and pH 10.0. However, for example, a preferred antigen-binding activity is an antigen-binding activity at a pH between pH 7.0 and pH 8.0, and a more preferred antigen-binding activity is an antigen-binding activity at pH 7.4. Further, the antigen-binding activity of the antigen-binding molecule at pH 4.0 to pH 6.5 is not particularly limited, as long as it is an antigen-binding activity at a pH between pH 4.0 and pH 6.5. However, for example, a preferred antigen-binding activity is an antigen-binding activity at a pH between pH 5.5 to pH 6.5, and a more preferred antigen-binding activity is an antigen-binding activity at pH 5.8 or pH 5.5.

**[0168]** The antigen-binding activity of an antigen-binding molecule can be determined by methods known to those skilled in the art. Conditions other than the pH can be appropriately determined by those skilled in the art. The antigen-binding activity of an antigen-binding molecule can be assessed as dissociation constant (KD), apparent dissociation constant (apparent KD), dissociation rate ( $k_d$ ), apparent dissociation rate (apparent  $k_d$ ), or such. These constants can be determined by methods known to those skilled in the art, for example, using Biacore (GE healthcare), Scatchard plot, or FACS.

**[0169]** Herein, "the step of selecting an antigen-binding molecule whose antigen-binding activity at pH 6.7 to pH 10.0 is greater than that at pH 4.0 to pH 6.5" has a same meaning as "the step of selecting an antigen-binding molecule whose antigen-binding activity at pH 4.0 to pH 6.5 is lower than that at pH 6.7 to pH 10.0".

**[0170]** The difference between the antigen-binding activity at pH 6.7 to pH 10.0 and that at pH 4.0 to pH 6.5 is not particularly limited as long as the antigen-binding activity at pH 6.7 to pH 10.0 is greater than that at pH 4.0 to pH 6.5. However, the antigen-binding activity at pH 6.7 to pH 10.0 is preferably twice or greater, more preferably ten times or greater, and still more preferably 40 times or greater than the antigen-binding activity at pH 4.0 to pH 6.5.

**[0171]** Furthermore, the present invention also provides methods of screening for antigen-binding molecules, which comprise the steps of:

- (a) binding an antigen-binding molecule to an antigen under a condition of pH 6.7 to pH 10.0;
- (b) placing the antigen-binding molecule that bound to the antigen of (a) under a condition of pH 4.0 to pH 6.5; and
- (c) obtaining the antigen-binding molecule that dissociated under the condition of pH 4.0 to pH 6.5.

5 **[0172]** In addition, the present invention also provides methods of screening for antigen-binding molecules, which comprise the steps of:

- (a) selecting an antigen-binding molecule that does not bind to an antigen under a condition of pH 4.0 to pH 6.5;
- (b) binding the antigen-binding molecule selected in (a) to an antigen under a condition of pH 6.7 to pH 10.0; and
- 10 (c) obtaining the antigen-binding molecule that bound to the antigen under the condition of pH 6.7 to pH 10.0.

**[0173]** Furthermore, the present invention also provides methods of screening for antigen-binding molecules, which comprise the steps of:

- 15 (a) binding an antigen-binding molecule to an antigen under a condition of pH 6.7 to pH 10.0;
- (b) placing the antigen-binding molecule that bound to the antigen of (a) under a condition of pH 4.0 to pH 6.5;
- (c) obtaining the antigen-binding molecule that dissociated under the condition of pH 4.0 to pH 6.5;
- (d) amplifying the gene encoding the antigen-binding molecule that dissociated; and
- (e) obtaining the eluted antigen-binding molecule.

20 **[0174]** The steps of (a) to (d) may be repeated twice or more times. Thus, the present invention provides the methods described above further including a step of repeating the steps of (a) to (d) twice or more times. The number for repeating the steps of (a) to (d) is not particularly limited; however, the number is in general ten or less.

25 **[0175]** Furthermore, the present invention also provides methods of screening for antigen-binding molecules, which comprise the steps of:

- (a) selecting an antigen-binding molecule that does not bind to an antigen under a condition of pH 4.0 to pH 6.5;
- (b) binding the antigen-binding molecule selected in (a) to an antigen under a condition of pH 6.7 to pH 10.0;
- (c) obtaining the antigen-binding molecule that bound to the antigen under the condition of pH 6.7 to pH 10.0;
- 30 (d) amplifying the gene encoding the antigen-binding molecule that dissociated; and
- (e) collecting the eluted antigen-binding molecule.

35 **[0176]** The steps of (a) to (d) may be repeated twice or more times. Thus, the present invention provides the methods described above further including a step of repeating the steps of (a) to (d) twice or more times. The number for repeating the steps of (a) to (d) is not particularly limited; however, the number is in general ten or less.

**[0177]** When a phage library or such is used in the screening methods of the present invention, the step of amplifying the gene encoding the antigen-binding molecule can also be a step of amplifying phages.

40 **[0178]** In the methods of the present invention, binding of the antigen-binding molecule and the antigen may be carried out under any state, without particular limitation. For example, binding of the antigen-binding molecule and the antigen may be carried out by contacting an antigen with an immobilized antigen-binding molecule, or by contacting an antigen-binding molecule with an immobilized antigen. Alternatively, binding of the antigen-binding molecule and the antigen may be carried out by contacting the antigen and antigen-binding molecule in a solution.

**[0179]** Furthermore, the present invention also provides methods of screening for antigen-binding molecules whose binding activity at a first pH is greater than that at a second pH, which comprise the steps of:

- 45 (a) binding an antigen-binding molecule to an antigen-immobilized column under the condition of a first pH;
- (b) eluting the antigen-binding molecule that had bound to the column at the first pH from the column under the condition of a second pH; and
- (c) obtaining the eluted antigen-binding molecule.

50 **[0180]** Furthermore, the present invention also provides methods of screening for antigen-binding molecules whose binding activity at a first pH is smaller than that at a second pH, which comprise the steps of:

- 55 (a) passing an antigen-binding molecule through an antigen-immobilized column under the condition of a first pH;
- (b) collecting the antigen-binding molecule that eluted without binding to the column in step (a);
- (c) binding the antigen-binding molecule collected in (b) to a column under the condition of a second pH; and
- (d) obtaining the antigen-binding molecule that bound to the column in step (c).



**[0181]** Furthermore, the present invention also provides methods of screening for antigen-binding molecules whose binding activity at a first pH is greater than that at a second pH, which comprise the steps of:

- (a) binding an antigen-binding molecule library to an antigen-immobilized column under the condition of a first pH;
- (b) eluting the antigen-binding molecule from the column under the condition of a second pH;
- (c) amplifying the gene encoding the eluted antigen-binding molecule; and
- (d) obtaining the eluted antigen-binding molecule.

**[0182]** The steps of (a) to (c) may be repeated twice or more times. Thus, the present invention provides the methods described above further including the step of repeating the steps of (a) to (c) twice or more times. The number for repeating the steps of (a) to (c) is not particularly limited; however, the number is in general ten or less.

**[0183]** In the present invention, each of the first and second pHs may be any pH, as long as they are not identical. In a preferred combination of the first and second pHs, for example, the first pH is between pH 6.7 and pH 10.0 and the second pH is between pH 4.0 and pH 6.5; in a more preferred combination, the first pH is between pH 7.0 and pH 8.0, and the second pH is between pH 5.5 and pH 6.5; and in a still more preferred combination, the first pH is pH 7.4 and the second pH is pH 5.8 or pH 5.5.

**[0184]** In another preferred combination of the first and second pHs, for example, the first pH is between pH 4.0 and pH 6.5, and the second pH is between pH 6.7 and pH 10.0; in a more preferred combination, the first pH is between pH 5.5 and pH 6.5, and the second pH is between pH 7.0 and pH 8.0; and in a still more preferred combination, the first pH is pH 5.8 or pH 5.5 and the second pH is pH 7.4.

**[0185]** Antigen-binding molecules that are screened by the methods of the present invention may be any antigen-binding molecules. For example, it is possible to use the above-described antigen-binding molecules in the screening of the present invention. For example, it is possible to screen antigen-binding molecules including natural sequences or antigen-binding molecules including amino acid sequences with substitutions. Preferred antigen-binding molecules that are screened in the present invention include, for example, antigen-binding molecules in which at least one amino acid is substituted with histidine or at least one histidine is inserted. The site of introduction of histidine substitution or insertion is not particularly limited, and may be introduced at any site. Furthermore, histidine substitution or insertion may be introduced at one site, or may be introduced at two or more sites. Furthermore, preferred antigen-binding molecules that are screened in the present invention include, for example, antigen-binding molecules including modified antibody constant regions.

**[0186]** Antigen-binding molecules that are screened by the methods of the present invention may be a number of different antigen-binding molecules introduced with histidine substitutions or insertions at different sites, for example, by histidine scanning.

**[0187]** Thus, the screening methods of the present invention may further comprise the step of substituting at least one amino acid in the antigen-binding molecule with histidine or inserting at least one histidine into the antigen-binding molecule.

**[0188]** In the screening methods of the present invention, non-natural amino acids may be used instead of histidine. Therefore, the present invention can also be understood by replacing the above-mentioned histidine with non-natural amino acids.

**[0189]** Moreover, the screening methods of the present invention may further comprise the step of modifying amino acids of antibody constant regions.

**[0190]** Antigen-binding substances that are screened by the screening methods of the present invention may be prepared by any method. For example, it is possible to use pre-existing antibodies, pre-existing libraries (phage libraries and the like), antibodies and libraries that are prepared from hybridomas obtained by immunizing animals or from B cells of immunized animals, antibodies and libraries (libraries with high content of histidine or non-natural amino acid, libraries introduced with histidine or non-natural amino acid at specific sites, and the like) prepared by introducing histidine mutations or non-natural amino acid mutations into the above-described antibodies and libraries, and so on.

**[0191]** Antigen-binding molecules that bind to the antigen multiple times, which are thus superior in the retention in plasma, can be obtained by the screening methods of the present invention. Thus, the screening methods of the present invention can be used as screening methods for obtaining antigen-binding molecules that are superior in the retention in plasma.

**[0192]** Furthermore, antigen-binding molecules that can bind to the antigen two or more times when administered to animals such as humans, mice, or monkeys can be obtained by the screening methods of the present invention. Thus, the screening methods of the present invention can be used as screening methods for obtaining antigen-binding molecules that can bind to the antigen two or more times.

**[0193]** Furthermore, antigen-binding molecules that are capable of binding to more antigens as compared to the number of their antigen-binding sites when administered to animals such as humans, mice, or monkeys can be obtained by the screening methods of the present invention. Thus, the screening methods of the present invention can be used

as screening methods for obtaining antigen-binding molecules that are capable of binding to more antigens as compared to the number of their antigen-binding sites. For example, when the antibody is a neutralizing antibody, the screening methods of the present invention can be used as screening methods for obtaining antigen-binding molecules that can neutralize more antigens as compared to the number of the antigen-binding sites of the antigen-binding molecules.

**[0194]** Furthermore, antigen-binding molecules that are capable of dissociating within a cell from an extracellularly-bound antigen when administered to animals such as humans, mice, or monkeys can be obtained by the screening methods of the present invention. Thus, the screening methods of the present invention can be used as screening methods for obtaining antigen-binding molecules that are capable of dissociating within a cell from an extracellularly-bound antigen.

**[0195]** Furthermore, antigen-binding molecules that are bound to an antigen and internalized into a cell, and released to the outside of the cell in an antigen-free form when administered to animals such as humans, mice, or monkeys can be obtained by the screening methods of the present invention. Thus, the screening methods of the present invention can be used as screening methods for obtaining antigen-binding molecules that are bound to an antigen and internalized into a cell, and released to the outside of the cell in an antigen-free form.

**[0196]** Furthermore, antigen-binding molecules that can rapidly eliminate antigens in plasma when administered to animals such as humans, mice, or monkeys can be obtained by the screening methods of the present invention. Thus, the screening methods of the present invention can be used as screening methods for obtaining antigen-binding molecules with increased (high) ability to eliminate antigens in plasma.

**[0197]** Furthermore, such antigen-binding molecules are expected to be especially superior as pharmaceuticals, because the dose and frequency of administration in patients can be reduced and as a result the total dosage can be reduced. Thus, the screening methods of the present invention can be used as methods of screening for antigen-binding molecules for use as pharmaceutical compositions.

**[0198]** In addition, the present invention provides libraries in which the histidine content is increased as compared to the original libraries. Libraries containing antigen-binding molecules with increased histidine content can be used in the screening methods described above and the production methods described hereinafter.

**[0199]** Libraries with increased histidine content can be prepared by methods known to those skilled in the art, which include the following method. 20 types of triplet codons (trinucleotides) encoding 20 types of amino acids can be incorporated at equal frequency when synthesizing nucleic acids to prepare a library by the trinucleotide-method (J Mol Biol. 2008 Feb 29; 376(4): 1182-200). As a result, the position mutated for the library can be made to contain 20 types of amino acids at equal probability. The frequency of histidine in the position mutated for the library can be increased by increasing the proportion of a histidine-encoding trinucleotide as compared to the remaining amino acids among the 20 types in the synthesis.

#### <Methods for producing antigen-binding molecules>

**[0200]** The present invention provides methods for producing antigen-binding molecules whose antigen-binding activity at the endosomal pH is lower than that at the plasma pH. The present invention also provides methods for producing antigen-binding molecules that are superior in the retention in plasma. The present invention also provides methods for producing antigen-binding molecules that are especially useful when used as pharmaceutical compositions.

**[0201]** Specifically, the present invention provides methods for producing antigen-binding molecules, which comprise the steps of:

- (a) determining the antigen-binding activity of an antigen-binding molecule at pH 6.7 to pH 10.0;
- (b) determining the antigen-binding activity of the antigen-binding molecule at pH 4.0 to pH 6.5;
- (c) selecting an antigen-binding molecule whose antigen-binding activity at pH 6.7 to pH 10.0 is greater than that at pH 4.0 to pH 6.5;
- (d) obtaining the gene encoding the antigen-binding molecule selected in (c); and
- (e) producing the antigen-binding molecule using the gene obtained in (d).

**[0202]** The present invention also provides methods for producing antigen-binding molecules, which comprise the steps of:

- (a) binding an antigen-binding molecule to an antigen at pH 6.7 to pH 10.0;
- (b) allowing the antigen-binding molecule bound to the antigen of (a) to stand under the condition of pH 4.0 to pH 6.5;
- (c) collecting the antigen-binding molecule that dissociated under the condition of pH 4.0 to pH 6.5;
- (d) obtaining the gene encoding the antigen-binding molecule obtained in (c); and
- (e) producing the antigen-binding molecule using the gene obtained in (d).

**[0203]** Furthermore, the present invention provides methods for producing antigen-binding molecules, which comprise the steps of:

- (a) selecting an antigen-binding molecule that does not bind to the antigen under the condition of pH 4.0 to pH 6.5;
- (b) binding the antigen under the condition of pH 6.7 to pH 10.0 to the antigen-binding molecule selected in (a);
- (c) collecting the antigen-binding molecule that bound to the antigen under the condition of pH 6.7 to pH 10.0;
- (d) obtaining the gene encoding the antigen-binding molecule collected in (c); and
- (e) producing the antigen-binding molecule using the gene obtained in (d).

**[0204]** In addition, the present invention provides methods for producing antigen-binding molecules, which comprise the steps of:

- (a) binding an antigen-binding molecule to an antigen under the condition of pH 6.7 to 10.0;
- (b) allowing the antigen-binding molecule that bound to the antigen in (a) to stand under the condition of pH 4.0 to pH 6.5;
- (c) collecting the antigen-binding molecule that dissociated under the condition of pH 4.0 to pH 6.5;
- (d) amplifying the gene encoding the dissociated antigen-binding molecule;
- (e) collecting the eluted antigen-binding molecule;
- (f) obtaining the gene encoding the antigen-binding molecule collected in (e); and
- (g) producing the antigen-binding molecule using the gene obtained in (f).

**[0205]** Steps (a) to (d) may be repeated twice or more times. Thus, the present invention provides the methods described above, which further comprise the step of repeating steps (a) to (d) twice or more times. The number of times steps (a) to (d) is repeated is not particularly limited; however, it is generally ten times or less.

**[0206]** Furthermore, the present invention provides methods of screening for antigen-binding molecules, which comprise the steps of:

- (a) selecting an antigen-binding molecule that does not bind to the antigen under the condition of pH 4.0 to pH 6.5;
- (b) binding the antigen under the condition of pH 6.7 to pH 10.0 to the antigen-binding molecule selected in (a);
- (c) collecting the antigen-binding molecule that bound to the antigen under the condition of pH 6.7 to pH 10.0;
- (d) amplifying the gene encoding the dissociated antigen-binding molecule;
- (e) collecting the eluted antigen-binding molecule;
- (f) obtaining the gene encoding the antigen-binding molecule collected in (e); and
- (g) producing the antigen-binding molecule using the gene obtained in (f).

**[0207]** Steps (a) to (d) may be repeated twice or more times. Thus, the present invention provides the methods described above, which further comprise the step of repeating steps (a) to (d) twice or more times. The number of times steps (a) to (d) is repeated is not particularly limited; however, it is generally ten times or less.

**[0208]** Furthermore, the present invention provides methods for producing antigen-binding molecules whose binding activity at a first pH is greater than that at a second pH, which comprise the steps of:

- (a) binding the antigen-binding molecule to a column immobilized with antigen under the first pH condition;
- (b) eluting the antigen-binding molecule, which is bound to the column under the first pH condition from the column under a second pH condition;
- (c) collecting the eluted antigen-binding molecule;
- (d) obtaining the gene encoding the antigen-binding molecule collected in (c); and
- (e) producing the antigen-binding molecule using the gene obtained in (d).

**[0209]** Furthermore, the present invention provides methods for producing antigen-binding molecules whose binding activity at a first pH is greater than that at a second pH, which comprise the steps of:

- (a) binding an antigen-binding molecule library to a column immobilized with antigen under the first pH condition;
- (b) eluting the antigen-binding molecule from the column under the second pH condition;
- (c) amplifying the gene encoding the eluted antigen-binding molecule;
- (d) collecting the eluted antigen-binding molecule;
- (e) obtaining the gene encoding the antigen-binding molecule collected in (d); and
- (f) producing the antigen-binding molecule using the gene obtained in (e).

**[0210]** Steps (a) to (c) may be repeated twice or more times. Thus, the present invention provides the methods described above, which further comprise the step of repeating steps (a) to (c) twice or more times. The number of times steps (a) to (c) is repeated is not particularly limited; however, it is generally ten times or less.

**[0211]** When a phage library or such is used in the production methods of the present invention, the step of amplifying the gene encoding the antigen-binding molecule may be the step of amplifying phages.

**[0212]** Antigen-binding substances that are used in the production methods of the present invention may be prepared by any method. For example, it is possible to use pre-existing antibodies, pre-existing libraries (phage libraries and the like), antibodies and libraries that are prepared from hybridomas obtained by immunizing animals or from B cells of immunized animals, antibodies and libraries (libraries with high content of histidine or non-natural amino acid, libraries introduced with histidine or non-natural amino acid at specific sites, and the like) prepared by introducing histidine mutations or non-natural amino acid mutations into the above-described antibodies and libraries, and so on.

**[0213]** In the above-described production methods, the antigen-binding activity of the antigen-binding molecule at pH 6.7 to pH 10.0 is not particularly limited, as long as the antigen-binding activity is that at a pH between pH 6.7 and pH 10.0. A preferred antigen-binding activity is that at a pH between pH 7.0 and pH 8.0, and a more preferred antigen-binding activity is that at pH 7.4. Alternatively, the antigen-binding activity of the antigen-binding molecule at pH 4.0 to pH 6.5 is not particularly limited, as long as the antigen-binding activity is that at a pH between pH 4.0 and pH 6.5. A preferred antigen-binding activity is that at a pH between pH 5.5 to pH 6.5, and a more preferred antigen-binding activity is that at pH 5.8 or pH 5.5.

**[0214]** The antigen-binding activity of an antigen-binding molecule can be determined by methods known to those skilled in the art. Conditions except for pH can be appropriately determined by those skilled in the art.

**[0215]** The step of selecting antigen-binding molecules whose antigen-binding activity at pH 6.7 to pH 10.0 is greater than that at pH 4.0 to pH 6.5 is synonymous with the step of selecting antigen-binding molecules whose antigen-binding activity at pH 4.0 to pH 6.5 is lower than that at pH 6.7 to pH 10.0.

**[0216]** The difference in the antigen-binding activity at pH 6.7 to pH 10.0 and at pH 4.0 to pH 6.5 is not particularly limited as long as the antigen-binding activity at pH 6.7 to pH 10.0 is greater than that at pH 4.0 to pH 6.5. The antigen-binding activity at pH 6.7 to pH 10.0 is preferably twice or greater, more preferably ten times or greater, and still more preferably 40 times or greater than that at pH 4.0 to pH 6.5.

**[0217]** In the production methods described above, the antigen-binding molecule may be bound to the antigen under any condition, and the condition is not particularly limited. For example, the antigen-binding molecule may be bound to the antigen by contacting the antigen with the immobilized antigen-binding molecule, or by contacting the antigen-binding molecule with the immobilized antigen. Alternatively, the antigen-binding molecule may be bound to the antigen by contacting the antigen and antigen-binding molecule in a solution.

**[0218]** In the production methods described above, each of the first and second pHs may be any pH, as long as they are not identical. In a preferred combination of the first and second pHs, for example, the first pH is between pH 6.7 and pH 10.0, and the second pH is between pH 4.0 and pH 6.5; in a more preferred combination, the first pH is between pH 7.0 and pH 8.0, and the second pH is between pH 5.5 and pH 6.5; and in a still more preferred combination, the first pH is pH 7.4 and the second pH is pH 5.8 or pH 5.5.

**[0219]** In another preferred combination of the first and second pHs, for example, the first pH is between pH 4.0 and pH 6.5, and the second pH is between pH 6.7 and pH 10.0; in a more preferred combination, the first pH is between pH 5.5 and pH 6.5, and the second pH is between pH 7.0 and pH 8.0; and in a still more preferred combination, the first pH is pH 5.8 or pH 5.5 and the second pH is pH 7.4.

**[0220]** Antigen-binding molecules that are produced by the production methods described above may be any antigen-binding molecules. Preferred antigen-binding molecules include, for example, antigen-binding molecules in which at least one amino acid is substituted with histidine or at least one histidine has been inserted. The site where such histidine mutation is introduced is not particularly limited and may be introduced at any site. Furthermore, histidine mutation may be introduced at one site or at two or more sites.

**[0221]** Thus, the production methods of the present invention may further comprise the step of substituting at least one amino acid in an antigen-binding molecule with histidine or inserting at least one histidine into antigen-binding molecules.

**[0222]** In the production methods of the present invention, non-natural amino acids may be used instead of histidine. Therefore, the present invention can also be understood by replacing the above-mentioned histidine with non-natural amino acids.

**[0223]** Furthermore, in another embodiment, the antigen-binding molecules that are produced by the production methods described above include, for example, antigen-binding molecules including modified antibody constant regions. Accordingly, the production methods of the present invention may further comprise the step of modifying the amino acids of antibody constant regions.

**[0224]** The antigen-binding molecules that are produced by the production methods of the present invention are superior in the retention in plasma. Thus, the production methods of the present invention can be used as methods for

producing antigen-binding molecules that are superior in the retention in plasma.

**[0225]** Furthermore, antigen-binding molecules produced by the production methods are expected to be capable of binding to the antigen two or more times when administered to animals such as humans, mice, or monkeys. Thus, the production methods of the present invention can be used as methods for producing antigen-binding molecules that are

**[0226]** Furthermore, antigen-binding molecules produced by the production methods of the present invention are expected to be capable of binding to more antigens as compared to the number of their antigen-binding sites when administered to animals such as humans, mice, or monkeys. Thus, the production methods of the present invention can be used as methods for producing antigen-binding molecules that are capable of binding to more antigens as compared

**[0227]** Furthermore, antigen-binding molecules produced by the production methods of the present invention are expected to be capable of dissociating within a cell from an extracellularly-bound antigen when administered to animals such as humans, mice, or monkeys. Thus, the production methods of the present invention can be used as methods for producing antigen-binding molecules that are capable of dissociating within a cell from an extracellularly-bound antigen.

**[0228]** Furthermore, antigen-binding molecules produced by the production methods of the present invention are expected to be capable of being bound to an antigen and internalized into a cell as well as being released to the outside of the cell in an antigen-free form, when administered to animals such as humans, mice, or monkeys. Thus, the production methods of the present invention can be used as methods for producing antigen-binding molecules that are capable of being bound to an antigen and internalized into a cell and being released to the outside of the cell in an antigen-free form.

**[0229]** Furthermore, antigen-binding molecules that are produced by the production methods of the present invention are expected to be capable of rapidly eliminating antigens from plasma when administered to animals such as humans, mice, or monkeys. Thus, the production methods of the present invention can be used as method four producing antigen-binding molecules with increased (high) ability to eliminate antigens in plasma.

**[0230]** Furthermore, such antigen-binding molecules can reduce the number of doses in patients and are expected to be especially superior as pharmaceuticals. Thus, the production methods of the present invention can be used as methods for producing antigen-binding molecules for used as pharmaceutical compositions.

**[0231]** Genes obtained by the production methods of the present invention are typically carried by (inserted into) appropriate vectors, and then introduced into host cells. The vectors are not particularly limited as long as they stably retain the inserted nucleic acids. For example, when *Escherichia coli* (*E. coli*) is used as the host, preferred cloning vectors include pBluescript vector (Stratagene); however, various commercially available vectors may be used. When using vectors to produce the antigen-binding molecules of the present invention, expression vectors are particularly useful. The expression vectors are not particularly limited as long as the vectors express the antigen-binding molecules *in vitro*, in *E. coli*, in culture cells, or in a body of an organism. For example, pBEST vector (Promega) is preferred for *in vitro* expression; pET vector (Invitrogen) is preferred for *E. coli*; pME18S-FL3 vector (GenBank Accession No. AB009864) is preferred for culture cells; and pME18S vector (Mol Cell Biol. 8:466-472 (1988)) is preferred for bodies of organisms. DNAs of the present invention can be inserted into the vectors by conventional methods, for example, by ligation using restriction enzyme sites (Current protocols in Molecular Biology, edit. Ausubel et al., (1987) Publish. John Wiley & Sons, Section 11.4-11.11).

**[0232]** The above host cells are not particularly limited, and various host cells may be used depending on the purpose. Examples of cells for expressing the antigen-binding molecules include bacterial cells (such as those of *Streptococcus*, *Staphylococcus*, *E. coli*, *Streptomyces*, and *Bacillus subtilis*), eukaryotic cells (such as those of yeast and *Aspergillus*), insect cells (such as *Drosophila* S2 and *Spodoptera* SF9), animal cells (such as CHO, COS, HeLa, C127, 3T3, BHK, HEK293, and Bowes melanoma cells), and plant cells. Vectors can be introduced into a host cell by known methods, for example, calcium phosphate precipitation methods, electroporation methods (Current protocols in Molecular Biology edit. Ausubel et al. (1987) Publish. John Wiley & Sons, Section 9.1-9.9), lipofection methods, and microinjection methods.

**[0233]** The host cells can be cultured by known methods. For example, when using animal cells as a host, DMEM, MEM, RPMI 1640, or IMDM may be used as the culture medium. They may be used with serum supplements such as FBS or fetal calf serum (FCS). The cells may be cultured in serum-free cultures. The preferred pH is about 6 to 8 during the course of culturing. Incubation is carried out typically at 30 to 40°C for about 15 to 200 hours. Medium is exchanged, aerated, or agitated, as necessary.

**[0234]** Appropriate secretion signals may be incorporated to polypeptides of interest so that the antigen-binding molecules expressed in the host cell are secreted into the lumen of the endoplasmic reticulum, into the periplasmic space, or into the extracellular environment.

**[0235]** These signals may be endogenous to the antigen-binding molecules of interest or may be heterologous signals.

**[0236]** On the other hand, for example, production systems using animals or plants may be used as systems for producing polypeptides *in vivo*. A polynucleotide of interest is introduced into an animal or plant and the polypeptide is produced in the body of the animal or plant, and then collected. The "hosts" of the present invention include such animals and plants.



[0237] The production system using animals include those using mammals or insects. It is possible to use mammals such as goats, pigs, sheep, mice, and bovines (Vicki Glaser SPECTRUM Biotechnology Applications (1993)). The mammals may be transgenic animals.

[0238] For example, a polynucleotide encoding an antigen-binding molecule of the present invention is prepared as a fusion gene with a gene encoding a polypeptide specifically produced in milk, such as the goat  $\beta$ -casein. Next, goat embryos are injected with polynucleotide fragments containing the fusion gene, and then transplanted to female goats. Desired antigen-binding molecules can be obtained from milk produced by the transgenic goats, which are born from the goats that received the embryos, or from their offspring. Hormones may be administered as appropriate to increase the volume of milk containing the antigen-binding molecule produced by the transgenic goats (Ebert et al., Bio/Technology (1994) 12: 699-702).

[0239] Insects such as silkworms may be used to produce the antigen-binding molecules of the present invention. When silkworms are used, baculoviruses carrying a polynucleotide encoding an antigen-binding molecule of interest can be used to infect silkworms, and the antigen-binding molecule of interest can be obtained from their body fluids.

[0240] Furthermore, when plants are used to produce the antigen-binding molecules of the present invention, for example, tobacco may be used. When tobacco is used, a polynucleotide encoding an antigen-binding molecule of interest is inserted into a plant expression vector, for example, pMON 530, and then the vector is introduced into bacteria, such as *Agrobacterium tumefaciens*. The bacteria are then allowed to infect tobacco such as *Nicotiana tabacum*, and the desired antigen-binding molecules can be collected from their leaves (Ma et al., Eur. J. Immunol. (1994) 24: 131-138). Alternatively, it is possible to infect duckweed (*Lemna minor*) with similar bacteria. After cloning, the desired antigen-binding molecules can be obtained from the duckweed cells (Cox KM et al., Nat. Biotechnol. 2006 Dec; 24(12): 1591-1597).

[0241] The thus obtained antigen-binding molecules may be isolated from the inside or outside (such as the medium and milk) of host cells, and purified as substantially pure and homogenous antigen-binding molecules. The methods for isolating and purifying antigen-binding molecules are not particularly limited, and isolation and purification methods usually used for polypeptide purification can be used. Antigen-binding molecules may be isolated and purified, by appropriately selecting and combining, for example, chromatographic columns, filtration, ultrafiltration, salting out, solvent precipitation, solvent extraction, distillation, immunoprecipitation, SDS-polyacrylamide gel electrophoresis, isoelectric focusing, dialysis, and recrystallization.

[0242] Chromatography includes, for example, affinity chromatography, ion exchange chromatography, hydrophobic chromatography, gel filtration, reverse-phase chromatography, and adsorption chromatography (Strategies for Protein Purification and Characterization: A Laboratory Course Manual. Ed Daniel R. Marshak et al., (1996) Cold Spring Harbor Laboratory Press). Such chromatographic methods can be conducted using liquid phase chromatography such as HPLC and FPLC. Columns used for affinity chromatography include, protein A columns and protein G columns. Columns using protein A include, for example, Hyper D, POROS, and Sepharose F. F. (Pharmacia).

[0243] If needed, an antigen-binding molecule can be modified arbitrarily, and peptides can be partially deleted by allowing an appropriate protein modification enzyme to act before or after purification of the antigen-binding molecule. Such protein modification enzymes include, for example, trypsin, chymotrypsin, lysyl endopeptidases, protein kinases, and glucosidases.

<Anti-IL-6 receptor antibodies>

[0244] Furthermore, the present invention provides the anti-IL-6 receptor antibodies of (a) to (m) below:

(a) an antibody that includes a heavy chain variable region including an amino acid sequence, in which at least one of Tyr at position 27, Asp at position 31, Asp at position 32, Trp at position 35, Tyr at position 51, Asn at position 59, Ser at position 63, Met at position 106, and Tyr at position 108 in the amino acid sequence of SEQ ID NO: 1 (H53 variable region) has been substituted with His;

(b) an antibody that includes a heavy chain variable region (H3pl) having an amino acid sequence, in which Tyr at position 27, Asp at position 31, and Trp at position 35 in the amino acid sequence of SEQ ID NO: 1 (H53 variable region) have been substituted with His;

(c) an antibody that includes a heavy chain variable region having an amino acid sequence, in which Tyr at position 27, Asp at position 31, Asp at position 32, Trp at position 35, Asn at position 59, Ser at position 63, and Tyr at position 108 in the amino acid sequence of SEQ ID NO: 1 (H53 variable region) have been substituted with His;

(d) an antibody that includes a heavy chain variable region (H170) having an amino acid sequence, in which Tyr at position 27, Asp at position 31, Asp at position 32, Trp at position 35, Asn at position 59, Ser at position 63, and Tyr at position 108 have been substituted with His, and in which Ser at position 99 has been substituted with Val and Thr at position 103 has been substituted with Ile in the amino acid sequence of SEQ ID NO: 1 (H53 variable region);

(e) an antibody that includes a heavy chain variable region having an amino acid sequence, in which Asp at position 31, Tyr at position 51, Ser at position 63, Met at position 106, and Tyr at position 108 in the amino acid sequence

of SEQ ID NO: 1 (H53 variable region) have been substituted with His;

(f) an antibody that includes a heavy chain variable region (CLH5) having an amino acid sequence, in which Asp at position 31, Tyr at position 51, Ser at position 63, Met at position 106, and Tyr at position 108 have been substituted with His, and in which Ser at position 99 has been substituted with Phe and Thr at position 103 has been substituted with Ile in the amino acid sequence of SEQ ID NO: 1 (H53 variable region);

(g) an antibody that includes a light chain variable region having an amino acid sequence, in which at least one of Asp at position 28, Tyr at position 32, Glu at position 53, Ser at position 56, and Asn at position 92 in the amino acid sequence of SEQ ID NO: 2 (PF1L variable region) has been substituted with His;

(h) an antibody that includes a light chain variable region (L73) having an amino acid sequence, in which Asp at position 28, Tyr at position 32, and Glu at position 53 in the amino acid sequence of SEQ ID NO: 2 (PF1L variable region) have been substituted with His;

(i) an antibody that includes a light chain variable region (L82) having an amino acid sequence, in which Tyr at position 32 and Glu at position 53 in the amino acid sequence of SEQ ID NO: 1 (H53 variable region) have been substituted with His;

(j) an antibody that includes a light chain variable region (CLL5) having an amino acid sequence, in which Tyr at position 32, Glu at position 53, Ser at position 56, and Asn at position 92 in the amino acid sequence of SEQ ID NO: 2 (PF1L variable region) have been substituted with His;

(k) an antibody that includes the heavy chain variable region of (b) and the light chain variable region of (h);

(l) an antibody that includes the heavy chain variable region of (d) and the light chain variable region of (i); and

(m) an antibody that includes the heavy chain variable region of (f) and the light chain variable region of (h).

**[0245]** Specific examples of the heavy chain variable region having an amino acid sequence in which at least one of Tyr at position 27, Asp at position 31, Asp at position 32, Trp at position 35, Tyr at position 51, Asn at position 59, Ser at position 63, Met at position 106, and Tyr at position 108 in the amino acid sequence of SEQ ID NO: 1 (H53 variable region) has been substituted with His include, for example, the following heavy chain variable regions.

a heavy chain variable region having the amino acid sequence of SEQ ID NO: 3 (H3pl)

a heavy chain variable region having the amino acid sequence of SEQ ID NO: 4 (H170)

a heavy chain variable region having the amino acid sequence of SEQ ID NO: 5 (CLH5)

**[0246]** Specific examples of the light chain variable region having an amino acid sequence in which at least one of Asp at position 28, Tyr at position 32, Glu at position 53, Ser at position 56, and Asn at position 92 in the amino acid sequence of SEQ ID NO: 2 (PF1L variable region) has been substituted with His include, for example, the following light chain variable regions.

a light chain variable region having the amino acid sequence of SEQ ID NO: 6 (L73)

a light chain variable region having the amino acid sequence of SEQ ID NO: 7 (L82)

a light chain variable region having the amino acid sequence of SEQ ID NO: 8 (CLL5)

**[0247]** The amino acid positions and substitutions in each of the above-described antibodies H3pl, H170, CLH5, L73, L82, and CLL5 are shown below in Table 1. The amino acid positions are shown based on the Kabat numbering.

[Table 1]

position	27	31	32	33	35	50	58	61	62	63	64	65	95	99	100B	102
H3pl	H	H		H	H											
H170	H	H	H	H	H		H		H				V	I		H
CLH5		H		H		H			H				F	I	H	H
position	24	27	28	32	53	55	56	90	92	94						
L73			H	H	H	H										
L82				H	H	H										
CLL5				H	H	H	H		H							
* In WT, the H chain has histidine at position 33, while the L chain has histidine at position 55.																

**[0248]** The present invention provides antibodies comprising at least any one of the amino acid substitutions described above in (a) to (j), and methods for producing the antibodies. Thus, the antibodies of the present invention also include antibodies comprising not only any of the amino acid substitutions described above in (a) to (j) but also amino acid



substitution(s) other than those described above in (a) to (j). Amino acid substitutions other than those described above in (a) to (j) include, for example, substitution, deletion, addition, and/or insertion in the amino acid sequence of CDRs and FRs.

**[0249]** Furthermore, the present invention provides the anti-IL-6 receptor antibodies of (1) to (28) below:

- (1) an antibody that includes the heavy chain variable region (VH1-IgG1 variable region) having the amino acid sequence from positions 1 to 119 in SEQ ID NO: 21 (VH1-IgG1);
- (2) an antibody that includes the heavy chain variable region (VH2-IgG1 variable region) having the amino acid sequence from positions 1 to 119 in SEQ ID NO: 22 (VH2-IgG1);
- (3) an antibody that includes the heavy chain variable region (VH3-IgG1 variable region) having the amino acid sequence from positions 1 to 119 in SEQ ID NO: 23 (VH3-IgG1);
- (4) an antibody that includes the heavy chain variable region (VH4-IgG1 variable region) having the amino acid sequence from positions 1 to 119 in SEQ ID NO: 24 (VH4-IgG1);
- (5) an antibody that includes the light chain variable region (VL1-CK variable region) having the amino acid sequence from positions 1 to 107 in SEQ ID NO: 25 (VL1-CK);
- (6) an antibody that includes the light chain variable region (VL2-CK variable region) having the amino acid sequence from positions 1 to 107 in SEQ ID NO: 26 (VL2-CK);
- (7) an antibody that includes the light chain variable region (VL3-CK variable region) having the amino acid sequence from positions 1 to 107 in SEQ ID NO: 27 (VL3-CK);
- (8) an antibody (FV1-IgG1) that includes the heavy chain variable region of (2) and the light chain variable region of (6);
- (9) an antibody (Fv2-IgG1) that includes the heavy chain variable region of (1) and a light chain variable region having the amino acid sequence of SEQ ID NO: 7 (L82);
- (10) an antibody (Fv3-IgG1) that includes the heavy chain variable region of (4) and the light chain variable region of (5);
- (11) an antibody (Fv4-IgG1) that includes the heavy chain variable region of (3) and the light chain variable region of (7);
- (12) an antibody (VH3-IgG2ΔGK) that includes a heavy chain having the amino acid sequence of SEQ ID NO: 33;
- (13) an antibody (VH3-M58) that includes a heavy chain having the amino acid sequence of SEQ ID NO: 34;
- (14) an antibody (VH3-M73) that includes a heavy chain having the amino acid sequence of SEQ ID NO: 35;
- (15) an antibody (Fv4-IgG2ΔGK) that includes the heavy chain of (12) and a light chain having the amino acid sequence of SEQ ID NO: 27 (VL3-CK);
- (16) an antibody (Fv4-M58) that includes the heavy chain of (13) and a light chain having the amino acid sequence of SEQ ID NO: 27 (VL3-CK);
- (17) an antibody (Fv4-M73) that includes the heavy chain of (14) and a light chain having the amino acid sequence of SEQ ID NO: 27 (VL3-CK);
- (18) an antibody (VH2-M71) that includes a heavy chain having the amino acid sequence of SEQ ID NO: 36 (VH2-M71);
- (19) an antibody (VH2-M73) that includes a heavy chain having the amino acid sequence of SEQ ID NO: 37 (VH2-M73);
- (20) an antibody (VH4-M71) that includes a heavy chain having the amino acid sequence of SEQ ID NO: 38 (VH4-M71);
- (21) an antibody (VH4-M73) that includes a heavy chain having the amino acid sequence of SEQ ID NO: 39 (VH4-M73);
- (22) an antibody (Fv1-M71) that includes the heavy chain of (18) and a light chain having the amino acid sequence of SEQ ID NO: 26 (VL2-CK);
- (23) an antibody (Fv1-M73) that includes the heavy chain of (19) and a light chain having the amino acid sequence of SEQ ID NO: 26 (VL2-CK);
- (24) an antibody (Fv3-M71) that includes the heavy chain of (20) and a light chain having the amino acid sequence of SEQ ID NO: 25 (VL1-CK);
- (25) an antibody (Fv3-M73) that includes the heavy chain of (21) and a light chain having the amino acid sequence of SEQ ID NO: 25 (VL1-CK);
- (26) an antibody that includes a light chain having the amino acid sequence of SEQ ID NO: 25 (VL1-CK);
- (27) an antibody that includes a light chain having the amino acid sequence of SEQ ID NO: 26 (VL2-CK); and
- (28) an antibody that includes a light chain having the amino acid sequence of SEQ ID NO: 27 (VL3-CK).

**[0250]** Furthermore, the present invention provides the FRs and CDRs of (a) to (v) below:

- (a) the heavy chain CDR1 of SEQ ID NO: 4:1 (VH1, 2, 3, 4);

- (b) the heavy chain CDR2 of SEQ ID NO: 41 (VH1, 2);
- (c) the heavy chain CDR2 of SEQ ID NO: 42 (VH3);
- (d) the heavy chain CDR2 of SEQ ID NO: 43 (VH4);
- (e) the heavy chain CDR3 of SEQ ID NO: 44 (VH1, 2);
- (f) the heavy chain CDR3 of SEQ ID NO: 45 (VH3, 4);
- (g) the heavy chain FR1 of SEQ ID NO: 46 (VH1, 2);
- (h) the heavy chain FR1 of SEQ ID NO: 47 (VH3, 4);
- (i) the heavy chain FR2 of SEQ ID NO: 48 (VH1, 2, 3, 4);
- (j) the heavy chain FR3 of SEQ ID NO: 49 (VH1);
- (k) the heavy chain FR3 of SEQ ID NO: 5 (VH2);
- (l) the heavy chain FR3 of SEQ ID NO: 51 (VH3, 4);
- (m) the heavy chain FR4 of SEQ ID NO: 52 (VH1, 2, 3, 4);
- (n) the light chain CDR1 of SEQ ID NO: 53 (VL1, 2);
- (o) the light chain CDR1 of SEQ ID NO: 54 (VL3);
- (p) the light chain CDR2 of SEQ ID NO: 55 (VL1, VL3);
- (q) the light chain CDR2 of SEQ ID NO: 56 (VL2);
- (r) the light chain CDR3 of SEQ ID NO: 57 (VL1, 2, 3);
- (s) the light chain FR1 of SEQ ID NO: 58 (VL1, 2, 3);
- (t) the light chain FR2 of SEQ ID NO: 59 (VL1, 2, 3);
- (u) the light chain FR3 of SEQ ID NO: 60 (VL1, 2, 3); and
- (v) the light chain FR4 of SEQ ID NO: 61 (VL1, 2, 3).

**[0251]** The respective sequences of the above (a) to (v) are shown in Fig. 25. Furthermore, the present invention provides polypeptides including any one of the FRs and CDRs of the above (a) to (v).

**[0252]** The anti-IL-6 receptor antibodies of the present invention also include fragments and modified products of antibodies including any of the amino acid substitutions described above. Such antibody fragments include, for example, Fab, F(ab')<sub>2</sub>, Fv, single chain Fv (scFv) in which Fv of H and L chains are linked together via an appropriate linker, single domain H chain and single domain L chain (for example, Nat. Biotechnol. 2005 Sep;23(9):1126-36), Unibody (WO 2007059782 A1), and SMIP (WO 2007014278 A2). The origin of antibodies is not particularly limited. The antibodies include human, mouse, rat, and rabbit antibodies. The antibodies of the present invention may also be chimeric, humanized, fully humanized antibodies, or such.

**[0253]** Specifically, such antibody fragments are obtained by treating antibodies with enzymes, for example, papain or pepsin, or by constructing genes that encode such antibody fragments, inserting them into expression vectors, and then expressing them in appropriate host cells (see, for example, Co, M.S. et al., J. Immunol. (1994) 152, 2968-2976; Better, M. & Horwitz, A. H. Methods in Enzymology (1989) 178, 476-496; Plueckthun, A. & Skerra, A. Methods in Enzymology (1989) 178, 497-515; Lamoyi, E., Methods in Enzymology (1989) 121, 652-663; Rousseaux, J. et al., Methods in Enzymology (1989) 121, 663-66; Bird, R. E. et al., TIBTECH (1991) 9, 132-137).

**[0254]** The present invention provides methods of producing (i) a polypeptide of the present invention, or (ii) a polypeptide encoded by a gene encoding the polypeptide of the present invention, wherein the methods comprise the step of culturing a host cell comprising a vector into which a polynucleotide encoding the polypeptide of the present invention is introduced.

**[0255]** More specifically, the present invention provides methods of producing a polypeptide of the present invention, which comprise the steps of:

- (a) culturing a host cell comprising a vector into which a gene encoding the polypeptide of the present invention is introduced; and
- (b) obtaining the polypeptide encoded by the gene.

**[0256]** scFv is obtained by linking the V regions of antibody H and L chains. In such scFv, the H chain V region is linked to the L chain V region via a linker, preferably a peptide linker (Huston, J. S. et al., Proc. Natl. Acad. Sci. U.S.A. (1988) 10.0, 5879-5883). The H chain and L chain V regions in an scFv may be derived from any of the antibodies described above. The peptide linker to link the V regions includes, for example, arbitrary single chain peptides of 12 to 19 amino acid residues.

**[0257]** When an anti-IL-6 receptor antibody of the present invention includes a constant region, the constant region may be of any type, for example, IgG1, IgG2, or IgG4 may be used. The constant region is preferably a human antibody constant region. Alternatively, the constant region may be a modified form including substitution, deletion, addition, and/or insertion in the amino acid sequence of human IgG1, human IgG2, or human IgG4 constant regions.

**[0258]** Preferred IL-6 receptor to which an anti-IL-6 receptor antibody of the present invention binds is human IL-6

receptor.

**[0259]** The anti-IL-6 receptor antibodies of the present invention are superior in the retention in plasma, and they exist for a prolonged period in the plasma in a form capable of binding to the antigen, i.e., soluble or membrane-associated IL-6 receptors. Thus, the anti-IL-6 receptor antibodies bind in vivo to soluble or membrane-associated IL-6 receptors for a prolonged period. Furthermore, the anti-IL-6 receptor antibodies are capable of binding to IL-6 receptors twice or more times, and thus assumed to be able to neutralize three or more IL-6 receptors.

#### <Pharmaceutical compositions>

**[0260]** The present invention also relates to pharmaceutical compositions that include antigen-binding molecules of the present invention, antigen-binding molecules isolated by the screening methods of the present invention, or antigen-binding molecules produced by the production methods of the present invention. The antigen-binding molecules of the present invention and antigen-binding molecules produced by the production methods of the present invention are superior in the retention in plasma, and thus, expected to reduce the administration frequency of the antigen-binding molecules, and are therefore useful as pharmaceutical compositions. The pharmaceutical composition of the present invention may include pharmaceutically acceptable carriers.

**[0261]** In the present invention, pharmaceutical compositions ordinarily refer to agents for treating or preventing, or testing and diagnosing diseases.

**[0262]** The pharmaceutical compositions of the present invention can be formulated by methods known to those skilled in the art. For example, they can be used parenterally, in the form of injections of sterile solutions or suspensions including water or other pharmaceutically acceptable liquid. For example, such compositions may be formulated by mixing in the form of unit dose required in the generally approved medicine manufacturing practice by appropriately combining with pharmaceutically acceptable carriers or media, specifically with sterile water, physiological saline, vegetable oil, emulsifier, suspension, surfactant, stabilizer, flavoring agent, excipient, vehicle, preservative, binder, or such. In such formulations, the amount of active ingredient is adjusted to obtain an appropriate amount in a pre-determined range.

**[0263]** Sterile compositions for injection can be formulated using vehicles such as distilled water for injection, according to standard formulation practice.

**[0264]** Aqueous solutions for injection include, for example physiological saline and isotonic solutions containing dextrose or other adjuvants (for example, D-sorbitol, D-mannose, D-mannitol, and sodium chloride). It is also possible to use in combination appropriate solubilizers, for example, alcohols (ethanol and such), polyalcohols (propylene glycol, polyethylene glycol, and such), non-ionic surfactants (polysorbate 80(TM), HCO-50, and such).

**[0265]** Oils include sesame oil and soybean oils. Bennyl benzoate and/or benzyl alcohol can be used in combination as solubilizers. It is also possible to combine buffers (for example, phosphate buffer and sodium acetate buffer), soothing agents (for example, procaine hydrochloride), stabilizers (for example, benzyl alcohol and phenol), and/or antioxidants.

Appropriate ampules are filled with the prepared injections.

**[0266]** The pharmaceutical compositions of the present invention are preferably administered parenterally. For example, the compositions may be in the dosage form for injections, transnasal administration, transpulmonary administration, or transdermal administration. For example, they can be administered systemically or locally by intravenous injection, intramuscular injection, intraperitoneal injection, subcutaneous injection, or such.

**[0267]** Administration methods can be appropriately selected in consideration of the patient's age and symptoms. The dose of a pharmaceutical composition containing an antigen-binding molecule may be, for example, from 0.0001 to 1000 mg/kg for each administration. Alternatively, the dose may be, for example, from 0.001 to 100,000 mg per patient. However, the present invention is not limited by the numeric values described above. The doses and administration methods vary depending on the patient's weight, age, symptoms, and such. Those skilled in the art can set appropriate doses and administration methods in consideration of the factors described above.

**[0268]** Amino acids contained in the amino acid sequences of the present invention may be post-translationally modified. For example, the modification of an N-terminal glutamine (Gln) residue into a pyroglutamic acid (pGlu) residue by pyroglutamylation is well-known to those skilled in the art. Naturally, such post-translationally modified amino acids are included in the amino acid sequences in the present invention.

**[0269]** All prior art documents cited in the specification are incorporated herein by reference.

#### Examples

**[0270]** Herein below, the present invention will be specifically described with reference to Examples, but it is not to be construed as being limited thereto.

## [Example 1] Production of Modified Humanized PM1 Antibody

Reparation of Recombinant Soluble Human IL-6 Receptor (SR344)

5 [0271] A recombinant human IL-6 receptor of the human IL-6 receptor, which served an antigen, was prepared as described below. A CHO cell line that constantly expresses a soluble human IL-6 receptor (hereinafter referred to as SR344) (Yamasaki, et al., Science 1988; 241: 825-828 (GenBank #X12830)) consisting of the amino acid sequence from the 1st amino acid to the 344th amino acid on the N-terminal side as reported in J. Biochem., 108, 673-676 (1990), was produced.

10 [0272] SR344 was purified from culture supernatant obtained from the SR344-expressing CHO cells using three column chromatographies: Blue Sepharose 6 FF column chromatography, affinity chromatography using a column in which an antibody specific to SR344 is immobilized, and gel filtration column chromatography. The fraction that eluted as the main peak was used as the final purified product.

15 Preparation of Recombinant Cynomolgus Monkey Soluble IL-6 Receptor (cIL-6R)

[0273] Oligo DNA primers Rhe6Rf1 (SEQ ID NO: 16) and Rhe6Rr2 (SEQ ID NO: 17) were produced based on the publicly-available rhesus monkey IL-6 receptor gene sequence (Birney et al., Ensemble 2006, Nucleic Acids Res., 2006, Jan. 1; 34 (Database issue): D556-61). Using cDNA prepared from cynomolgus pancreas as a template, a DNA fragment  
20 encoding the entire length of cynomolgus monkey IL-6 receptor gene was prepared by PCR using primers Rhe6Rf1 and Rhe6Rr2. Using the resulting DNA fragment as a template, a 1131 bp DNA fragment (SEQ ID NO: 20) encoding a protein in which 6xHis is added to the C terminal of the soluble region (Met1-Pro363) containing a signal region of cynomolgus monkey IL-6 receptor gene, was amplified by PCR using the oligo DNA primers CynoIL6R N-EcoRI (SEQ ID NO: 18) and CynoIL6R C-NotI-His (SEQ ID NO: 19). The resulting DNA fragment was digested with EcoRI and NotI and inserted  
25 into a mammalian cell expression vector, and this was then used to produce a stable expression CHO line (cyno.sIL-6R-producing CHO cells).

[0274] A culture medium of cyno.sIL-6R-producing CHO cells was purified with a HisTrap column (GE Healthcare Biosciences), concentrated using Amicon Ultra-15 Ultracel-10k (Millipore), and further purified with a Superdex 200 pg 16/60 gel filtration column (GE Healthcare Biosciences) to obtain the final purified product of soluble cynomolgus monkey  
30 IL-6 receptor (hereinafter referred to as cIL-6R).

Preparation of Recombinant Cynomolgus Monkey IL-6 (cIL-6)

[0275] Cynomolgus monkey IL-6 was prepared as follows. A nucleotide sequence encoding the 212 amino acids  
35 registered under SWISSPROT Accession No. P79341 was produced, cloned into a mammalian cell expression vector, and introduced into CHO cells to produce a stable expression cell line (cyno.IL-6-producing CHO cells). A culture medium of cyno.IL-6-producing CHO cells was purified with an SP-Sepharose/FF column (GE Healthcare Biosciences), concentrated using Amicon Ultra-15 Ultracel-5k (Millipore), and then further purified with a Superdex 75 pg 26/60 gel filtration column (GE Healthcare Biosciences). This was concentrated using Amicon Ultra-15 Ultracel-5k (Millipore) to obtain a  
40 final purified product of cynomolgus monkey IL-6 (hereinafter referred to as cIL-6).

Establishment of Human gp130-Expressing BaF3 Cell Line

45 [0276] A BaF3 cell line expressing human gp130 was established as indicated below in order to obtain a cell line exhibiting IL-6-dependent growth.

[0277] Full-length human gp130 cDNA (Hibi et al., Cell 1990; 63: 1149-1157 (GenBank #NM\_002184)) was amplified by PCR and cloned into the expression vector pCOS2Zeo, which was prepared by removing the DHFR gene expression site from pCHO1 (Hirata, et al., FEBS Letter 1994; 356: 244-248) and inserting a Zeocin resistance gene expression site, to construct pCOS2Zeo/gp130. Full-length human IL-6R cDNA was amplified by PCR and cloned into pcDNA3.1  
50 (+) (Invitrogen) to construct hIL-6R/pcDNA3.1(+). 10 µg of pCOS2Zeo/gp130 was mixed into BaF3 cells ( $0.8 \times 10^7$  cells) suspended in PBS, and pulsed using a Gene Pulser (Bio-Rad) at a voltage of 0.33 kV and capacitance of 950 µFD. BaF3 cells having undergone gene introduction by electroporation treatment were cultured one whole day and night in RPMI1640 medium (Invitrogen) containing 0.2 ng/mL of mouse interleukin-3 (Peprotech) and 10% fetal bovine serum (hereinafter referred to as FBS, HyClone), and then screened by adding RPMI1640 medium containing 100 ng/mL of  
55 human interleukin-6 (R&D Systems), 100 ng/mL of human interleukin-6 soluble receptor (R&D Systems) and 10% FBS to establish a human gp130-expressing BaF3 cell line (hereinafter referred to as BaF3/gp130). Since this BaF3/gp130 proliferates in the presence of human interleukin-6 (R&D Systems) and SR344, it can be used to evaluate the growth inhibitory activity (namely, IL-6 receptor-neutralizing activity) of anti-IL-6 receptor antibody.

Production of Humanized Anti-IL-6 Receptor Antibody

[0278] In the context of the instant example and elsewhere herein, the term "wild type" is abbreviated as WT, the term "wild type H chain" is abbreviated as H(WT) (amino acid sequence of SEQ ID NO: 9), and the term "wild type L chain" is abbreviated as L(WT) (amino acid sequence: SEQ ID NO: 10). In this context, mutations were introduced into the framework sequence and CDR sequence of humanized mouse PM1 antibody described in Cancer Res. 1993, Feb. 15; 53(4): 851-6, to produce modified H chains H53 (amino acid sequence: SEQ ID NO: 1) and PF1H (amino acid sequence: SEQ ID NO: 11), and modified L chains L28 (amino acid sequence: SEQ ID NO: 12) and PF1L (amino acid sequence: SEQ ID NO: 2). More specifically, the mutants were produced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the method described in the instructions provided, and the resulting plasmid fragments were inserted into a mammalian cell expression vector to produce the desired H chain expression vectors and L chain expression vectors. The nucleotide sequences of the obtained expression vectors were determined using conventional methodologies known to persons skilled in the art.

Expression and Purification of Humanized Anti-IL-6 Receptor Antibody

[0279] The antibodies were expressed by the method described below. Human embryonic kidney cancer-derived HEK293H cell line (Invitrogen) was suspended in DMEM (Invitrogen) supplemented with 10% Fetal Bovine Serum (Invitrogen). The cells were plated at 10 ml per dish in dishes for adherent cells (10 cm in diameter; CORNING) at a cell density of 5 to 6 x 10<sup>5</sup> cells/ml and cultured in a CO<sub>2</sub> incubator (37°C, 5% CO<sub>2</sub>) for one whole day and night. Then, the medium was removed by aspiration, and 6.9 ml of CHO-S-SFM-II medium (Invitrogen) was added. The prepared plasmid was introduced into the cells by the lipofection method. The resulting culture supernatants were collected, centrifuged (approximately 2000 g, 5 min, room temperature) to remove cells, and sterilized by filtering through 0.22-μm filter MILLEX®-GV (Millipore) to obtain the supernatants. Antibodies were purified from the obtained culture supernatants by a method known to those skilled in the art using rProtein A Sepharose™ Fast Flow (Amersham Biosciences). To determine the concentration of the purified antibody, absorbance was measured at 280 nm using a spectrophotometer. Antibody concentrations were calculated from the determined values using an absorbance coefficient calculated by the PACE method (Protein Science 1995; 4:2411-2423).

[Example 2] Production of pH-Dependently-Binding Antibody H3pl/L73Method for Creating Antibody Capable of Neutralizing Antigen Multiple Times

[0280] Since IgG molecules are divalent, a single IgG molecule can neutralize up to two antigen molecules when the two sites bind to the antigens; however, it cannot neutralize three or more antigen molecules. Therefore, to maintain the neutralizing effect of a neutralizing antibody over a certain period, it is necessary to administer an amount of the antibody equal to or greater than the amount of antigen produced during the period. Thus, there is a limitation on the extent to which the required dose of antibody can be reduced by improving the pharmacokinetics or affinity of antibody. Therefore, if it were possible to neutralize two or more antigen molecules with a single IgG molecule, the same dose could improve the duration of neutralizing effect, or alternatively the dose of antibody required to achieve the same duration could be reduced.

[0281] For neutralizing antibodies, there are two types of target antigens: soluble-type antigens, which are present in plasma, and membrane-bound antigens, which are expressed on the surface of cells.

[0282] When the antigen is a membrane-bound antigen, an administered antibody binds to the membrane antigen on the cellular surface, and the antibody is subsequently taken up into endosomes within the cell by internalization together with the membrane antigen bound to the antibody. Then, the antibody which is kept bound to the antigen moves to a lysosome where it is degraded by lysosome together with the antigen. The elimination of antibody from the plasma mediated by internalization by membrane antigen is referred to as antigen-dependent elimination, and this has been reported for numerous antibody molecules (Drug Discov. Today, 2006 Jan; 11(1-2): 81-8). Since a single IgG antibody molecule binds to two antigen molecules when it divalently binds to antigens, and is then internalized and directly degraded by lysosome, a single ordinary IgG antibody cannot neutralize two or more antigen molecules (Fig. 1).

[0283] The reason for the long retention (slow elimination) of IgG molecules in plasma is that FcRn, known as an IgG molecule salvage receptor, functions (Nat. Rev. Immunol. 2007 Sep; 7(9): 715-25). IgG molecules that have been taken up into endosomes by pinocytosis bind to FcRn expressed in endosomes under intraendosomal acidic conditions. IgG molecules bound to FcRn move to the cell surface where they dissociate from FcRn under neutral conditions in plasma and return to plasma, while IgG molecules unable to bind to FcRn proceed into lysosomes where they are degraded (Fig. 2).

[0284] IgG molecules bound to a membrane antigen are taken up into intracellular endosomes by internalization,



move into lysosomes while bound to the antigen, and undergo degradation. When an IgG antibody divalently binds to antigens, it neutralizes two antigen molecules and undergoes degradation together with the antigens. If the IgG antibody, when taken up into intracellular endosomes by internalization, can dissociate from the antigen under intraendosomal acidic conditions, the dissociated antibody may be able to bind to FcRn expressed in the endosomes. The IgG molecule dissociated from the antigen and bound to FcRn is transferred to the cell surface and then dissociated from FcRn under neutral conditions in the plasma, thereby return to the plasma again. The IgG molecule that has returned to the plasma is able to bind to a new membrane antigen again. The repetition of this process allows a single IgG molecule to repeatedly bind to membrane antigens, thereby enabling neutralization of a multiple antigens with a single IgG molecule (Fig. 3).

**[0285]** In the case of a soluble antigen, an antibody administered binds to the antigen in the plasma, and remains in the plasma in the form of an antigen-antibody complex. Normally, while the retention of antibody in plasma is very long (elimination rate is very slow) due to the function of FcRn as described above, the retention of antigen in plasma is short (elimination rate is fast). Thus, antibody-bound antigens show retention in plasma comparable to that of antibody (elimination rate is very slow). Antigens are produced in the body at a constant rate and, in the absence of antibody, present in plasma at a concentration at which the antigen production rate and the antigen elimination rate are under equilibrium. In the presence of antibody, most of the antigens are bound to antibodies, resulting in the very slow elimination of antigens. Thus, the antigen concentration in plasma increases as compared with that in the absence of antibody (Kidney Int. 2003, 64, 697-703; J. National Cancer Institute 2002, 94(19), 1484-1493; J. Allergy and Clinical Immunology 1997, 100(1), 110-121; Eur. J. Immunol. 1993, 23; 2026-2029). Even if the affinity of antibody for antigen is infinite, antigen concentration elevates as antibody is slowly eliminated from the plasma, and the neutralizing effect of antibody terminates after the concentrations of antibody and antigen become equal. Although antibodies with a stronger dissociation constant (KD) can neutralize soluble antigens at a lower antibody concentration, antibodies at a concentration half or less than the concentration of antigen present are unable to neutralize antigens regardless of how strong the affinity of antibody is (Biochem. Biophys. Res. Commun. 2005 Sep 9; 334(4): 1004-13). As is the case with IgG molecules not bound to antigens, IgG molecules bound to antigens in the plasma are also taken up into endosomes by pinocytosis, and bind to FcRn expressed in endosomes under intraendosomal acidic conditions. The IgG molecules bound to FcRn moves to the cell surface while the antibody is kept bound to the antigen and then dissociate from the FcRn under neutral conditions in the plasma. Since the IgG molecules return to the plasma while bound to the antigen, they cannot bind to new antigens in the plasma. In this case, if IgG molecules can dissociate from the antigen under intraendosomal acidic conditions, the dissociated antigen will not be able to bind to FcRn and thereby may be degraded by lysosomes. On the other hand, the IgG molecules can return to the plasma again by binding to FcRn. Since the IgG molecules that have returned to the plasma have already dissociated from the antigen in endosomes, they are able to bind to a new antigen again in the plasma. The repetition of this process allows a single IgG molecule to repeatedly bind to soluble antigens. This enables a single IgG molecule to neutralize multiple antigens (Fig. 4).

**[0286]** Thus, regardless of whether the antigen is a membrane antigen or soluble antigen, if the dissociation of IgG antibody from the antigen is possible under intraendosomal acidic conditions, a single IgG molecule would be able to repeatedly neutralize antigens. In order for IgG antibodies to dissociate from antigens under intraendosomal acidic conditions, it is necessary that antigen-antibody binding be considerably weaker under acidic conditions than under neutral conditions. Since membrane antigens on the cell surface need to be neutralized, antibodies have to strongly bind to antigens at the cell surface pH, namely pH 7.4. Since the intraendosomal pH has been reported to be typically pH 5.5 to 6.0 (Nat. Rev. Mol. Cell. Biol. 2004 Feb; 5(2): 121-32), an antibody that weakly binds to an antigen at pH 5.5 to 6.0 is considered to dissociate from the antigen under intraendosomal acidic conditions. More specifically, a single IgG molecule that strongly binds to an antibody at the cell surface pH of 7.4 and weakly binds to the antigen at the intraendosomal pH of 5.5 to 6.0 may be able to neutralize a multiple antigens and thereby improve the pharmacokinetics.

**[0287]** In general, protein-protein interactions consist of hydrophobic interaction, electrostatic interaction and hydrogen bonding, and the binding strength is typically expressed as a binding constant (affinity) or apparent binding constant (avidity). pH-dependent binding, whose binding strength varies between neutral conditions (pH 7.4) and acidic conditions (pH 5.5 to 6.0), is present in naturally-occurring protein-protein interactions. For example, the above-mentioned binding between IgG molecules and FcRn known as a salvage receptor for IgG molecules is strong under acidic conditions (pH 5.5 to 6.0) but remarkably weak under neutral conditions (pH 7.4). Most of such pH-dependently changing protein-protein interactions are associated with histidine residues. Since the pKa of histidine residue is in the vicinity of pH 6.0 to 6.5, the proton dissociation state of histidine residues varies between neutral conditions (pH 7.4) and acidic conditions (pH 5.5 to 6.0). Specifically, histidine residues are not charged and function as hydrogen atom acceptors under neutral conditions (pH 7.4), while they become positively charged and function as hydrogen atom donors under acidic conditions (pH 5.5 to 6.0). It has been reported that the pH-dependent binding of the above-described IgG-FcRn interaction is also associated with histidine residues present in IgG (Mol. Cell. 2001 Apr; 7(4): 867-77).

**[0288]** Therefore, pH-dependence can be imparted to protein-protein interactions by substituting an amino acid residue involved in protein-protein interactions with a histidine residue, or by introducing a histidine into an interaction site. Such attempts have also been made in protein-protein interactions between antibodies and antigens, and a mutant antibody

with antigen-binding ability decreased under acidic conditions has been successfully acquired by introducing histidine into the CDR sequence of an anti-egg white lysozyme antibody (FEBS Letter (vol. 309, No. 1, 85-88, 1992)). In addition, an antibody that is prepared by introducing histidine into its CDR sequence and specifically binds to an antigen at the low pH of cancer tissues but weakly binds under neutral conditions has been reported (WO 2003-105757).

**[0289]** Although methods for introducing pH dependency into antigen-antibody reactions have been reported as described above, an IgG molecule that neutralizes multiple antigens by strongly binding to antigens at the body fluid pH of 7.4 but weakly binding to antigens at the intraendosomal pH of pH 5.5 to 6.0 has not been reported. In other words, there have been no reports relating to modifications that significantly reduce the binding under acidic conditions while maintaining the binding under neutral conditions such that, as compared to an unmodified antibody, a modified antibody binds to antigens multiple times *in vivo* and thereby shows improved pharmacokinetics as well as improved duration of the neutralizing effect at the same dose.

**[0290]** The IL-6 receptor is present in the body in the form of either soluble IL-6 receptor or membrane IL-6 receptor (Nat. Clin. Pract. Rheumatol. 2006 Nov; 2(11): 619-26). Anti-IL-6 receptor antibodies bind to both the soluble IL-6 receptor and membrane IL-6 receptor, and neutralize their biological action. It is considered that, after binding to the membrane IL-6 receptor, anti-IL-6 receptor antibodies are taken up into intracellular endosomes by internalization while bound to the membrane IL-6 receptor, then move into lysosomes while the antibodies are kept bound to the membrane IL-6 receptor, and undergo degradation by lysosomes together with the membrane IL-6 receptor. In fact, it has been reported that a humanized anti-IL-6 receptor antibody exhibits non-linear clearance, and its antigen-dependent elimination greatly contributes to the elimination of the humanized anti-IL-6 receptor antibody (The Journal of Rheumatology, 2003, 30; 71426-1435). Thus, one humanized anti-IL-6 receptor antibody binds to one or two membrane IL-6 receptors (monovalently or divalently), and is then internalized and degraded in lysosomes. Therefore, if it is possible to produce modified antibodies that exhibit greatly reduced binding ability under acidic conditions but retain the same binding ability as the wild type humanized anti-IL-6 receptor antibody under neutral conditions (pH-dependent binding anti-IL-6 receptor antibody), multiple IL-6 receptors can be neutralized with a single humanized anti-IL-6 receptor antibody. Thus, in comparison with wild type humanized anti-IL-6 receptor antibodies, pH-dependent binding anti-IL-6 receptor antibodies may improve the duration of the neutralizing effect *in vivo* at the same dosage.

#### Production of pH-Dependently Binding Humanized Anti-IL-6 Receptor Antibody H3pl/L73:

**[0291]** Introduction of histidine into a CDR has been reported as a method for introducing pH-dependent binding to antigen-antibody reaction (FEBS Letter (vol. 309, No. 1, 85-88, 1992)). In order to find amino acid residues exposed on the surface of the variable region of the H53/PF1L produced in Example 1 and possible residues interacting with the antigen, a Fv region model of H53/PF1L was created by homology modeling using MOE software (Chemical Computing Group Inc.). A three-dimensional model constructed on the basis of the sequence information of H53/PF1L was used to select H27, H31, H35, L28, L32 and L53 (Kabat numbering, Karat, E.A. et al., 1991, Sequences of Proteins of Immunological Interest, NIH) as mutation sites that may introduce pH-dependent antigen-binding by histidine introduction. The product in which the residues at H27, H31 and H35 in H53 produced in Example 1 were substituted with histidines was designated as H3pl (amino acid sequence: SEQ ID NO: 3), and the product in which the residues at L28, L32 and L53 in PF1L produced in Example 1 were substituted with histidines was designated as L73 (amino acid sequence: SEQ ID NO: 6).

#### Production, Expression and Purification of H3pl/L73 Expression Vector

**[0292]** Amino acid modification was carried out to produce antibodies modified at the selected sites. Mutations were introduced into H53 (nucleotide sequence: SEQ ID NO: 13) and PF1L (nucleotide sequence: SEQ ID NO: 14) produced in Example 1 to produce H3pl (amino acid sequence: SEQ ID NO: 3) and L73 (amino acid sequence: SEQ ID NO: 6). More specifically, the QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used according to the method described in the instructions provided, and the resulting plasmid fragments were inserted into a mammalian cell expression vector to produce the desired H chain expression vector and L chain expression vector. The nucleotide sequences of the resulting expression vectors were determined using a method known to persons skilled in the art. H3pl/L73 which uses H3pl for the H chain and L73 for the L chain was expressed and purified by the method described in Example 1.

[Example 3] Conferring pH-Dependent Antigen Binding Ability by His Modification of CDR Using Phage Display Technology

#### Production of scFv Molecule of Humanized PM1 Antibody

**[0293]** The humanized PM1 antibody, which is a humanized anti-IL-6R antibody (Cancer Res. 1993 Feb 15; 53(4):

851-6), was converted into scFv. The VH and VL regions were amplified by PCR, and humanized PM1 HL scFv having the linker sequence GGGGSGGGGSGGGGS (SEQ ID NO. 15) between VH and VL was produced.

#### Selection of Histidine-Introducible Positions by Histidine Scanning

**[0294]** PCR was performed using the produced humanized PM1 HL scFv DNA as a template to produce a histidine library in which any one of the CDR amino acids is replaced with histidine. The library portions were constructed by PCR using primers in which the codon of an amino acid desired to be mutated for the library was replaced with CAT, a codon corresponding to histidine, and other portions were constructed by normal PCR. These portions were then linked by assemble PCR. The constructed library was digested with SfiI, inserted into a phagemide vector pELBG lacI that was also digested with SfiI, and then used to transform XL1-Blue (Stratagene). The resulting colonies were used to evaluate antigen binding by phage ELISA and analyze the sequence of HL scFv. Phage ELISA was carried out using a plate coated with SR344 at 1 µg/mL in accordance with J. Mol. Biol. 1992; 227: 381-388. Clones that were found to bind to SR344 were subjected to sequence analysis using specific primers.

**[0295]** Phage titer was determined by ELISA with an anti-Etag antibody (GE Healthcare) and anti-M13 antibody (GE Healthcare). This value was then used to select positions where substitution of the CDR residue with histidine did not significantly alter the binding ability as compared to humanized PM1 HL scFv, based on the results of phage ELISA for SR344. The selected positions are shown in Table 2. Numbering of each residue was in accordance with the Kabat numbering (Kabat, et al., 1991, Sequences of Proteins of Immunological Interest, NIH).

[Table 2] Positions of Histidine Substitution Not Significantly Affecting Binding Ability  
H31, H50, H54, H56, H57, H58, H59, H60, H61, H62, H63, H64, H65, H100a, H100b, H102  
L24, L26, L27, L28, L30, L31, L32, L52, L53, L54, L56, L90, L92, L93, L94

#### Construction of Histidine-Modified CDR Library

**[0296]** A library was designed in which the amino acids of CDR residues that did not significantly alter the binding ability when substituted with histidine as shown in Table 2 (histidine-introducible positions) are their original sequence (wild type sequence) or histidine. The library was constructed based on the sequences of the H chain PF1H and the L chain PF1L produced in Example 1 such that the mutated positions for the library have the original sequences or histidines (either the original sequence or histidines).

**[0297]** The library portions were constructed by PCR using primers that were designed such that a position desired to be mutated for the library has the original amino acid codon or histidine codon, and other portions were produced by normal PCR, or by PCR using synthetic primers as in the library portions. These portions were then linked by assemble PCR (J. Mol. Biol. 1996; 256: 77-88).

**[0298]** This library was used to construct a ribosome display library in accordance with J. Immunological Methods 1999; 231: 119-135. In order to carry out *Escherichia coli* cell-free *in vitro* translation, an SDA sequence (ribosome binding site) and T7 promoter were added to the 5' side, and a gene3 partial sequence serving as a linker for ribosome display was ligated to the 3' side using SfiI.

#### Acquisition of pH-Dependent Binding scFv from Library by Bead Panning

**[0299]** In order to concentrate only scFv having the ability to bind to SR344, panning was carried out twice by the ribosome display method in accordance with Nature Biotechnology 2000 Dec; 18: 1287-1292. The prepared SR344 was biotinylated using NHS-PE04-Biotin (Pierce) to obtain an antigen. Panning was carried out using 40 nM of the biotinylated antigen.

**[0300]** Using the resulting DNA pool as a template, HL scFv was restored by PCR using specific primers. After digesting with SfiI, the digested HL scFv was inserted into a phagemide vector pELBG lacI that was also digested with SfiI, and then used to transform XL1-Blue (Stratagene).

**[0301]** *Escherichia coli* cells carrying the desired plasmid were grown to 0.4 to 0.6 O.D./mL in 2YT medium containing 100 µg/mL ampicillin and 2% glucose. A helper phage (M13K07, 4.5 x 10<sup>11</sup> pfu) was added thereto, statically cultured for 30 minutes at 37°C, and then cultured with shaking for 30 minutes at 37°C. The culture was transferred to a 50 mL Falcon tube, centrifuged for 10 minutes at 3000 rpm, resuspended in 2YT medium containing 100 µg/mL ampicillin, 25 µg/mL kanamycin, and 0.5 mM IPTG, and then incubated overnight at 30°C.

**[0302]** The culture incubated overnight was precipitated with 2.5 M NaCl and 10% PEG, and then diluted with PBS to obtain a phage library solution. 10% M-PBS (PBS containing 10% skim milk) and 1 M Tris-HCl were added to the phage library solution to the final concentration of 2.5% M-PBS and pH 7.4. Panning was carried out by a typical panning

method using an antigen immobilized on magnetic beads (J. Immunol. Methods 2008 Mar 20; 332(1-2): 2-9; J. Immunol. Methods 2001 Jan 1; 247(1-2): 191-203; Biotechnol. Prog. 2002 Mar-Apr; 18(2): 212-20). More specifically, 40 pmol of biotin-labeled SR344 was added to the prepared phage library and the library was contacted with the antigen for 60 minutes at 37°C. Streptavidin-coated beads (DynaM-280) washed with 5% M-PBS (PBS containing 5% skim milk) were added and allowed to bind for 15 minutes at 37°C. The beads were washed five times with both 0.5 ml of PBST (PBS containing 0.1% Tween-20, pH 7.4) and PBS (pH 7.4). The beads were then suspended in 1 mL of PBS (pH 5.5) at 37°C, and the phage was recovered immediately. The recovered phage solution was added to 10 mL of logarithmic-growth-phase XL1-Blue (OD<sub>600</sub> of 0.4 to 0.5) and allowed to stand for 30 minutes at 37°C for infection. The infected *E. coli* were plated onto a 225 mm x 225 mm plate containing 2 YT, 100 µg/mL ampicillin and 2% glucose. These *E. coli* were used to begin additional phage culture in the same manner as described above and repeat the panning 8 times.

#### Evaluation by Phage ELISA

**[0303]** The above single colonies were inoculated in 100 µL of 2YT, 100 µg/mL ampicillin, 2% glucose and 12.5 µg/mL tetracycline and cultured overnight at 30°C. 2 µL of this culture was inoculated into 300 µL of 2YT, 100 µg/mL ampicillin and 2% glucose, and then cultured for 4 hours at 37°C. A helper phage (M13KO7) was added to the culture at  $9 \times 10^8$  pfu, allowed to stand for 30 minutes at 37°C and then shaken for 30 minutes at 37°C for infection. Subsequently, the medium was replaced with 300 µL of 2 YT, 100 µg/mL ampicillin, 25 µg/mL kanamycin, and 0.5 mM IPTG. After culturing overnight at 30°C, the centrifuged supernatant was recovered. 360 µL of 50 mM PBS (pH 7.4) was added to 40 µL of the centrifuged supernatant and subjected to ELISA. A Strepta Well 96-well microtiter plate (Roche) was coated overnight with 100 µL of PBS containing 62.5 ng/mL of biotin-labeled SR344. After removing the antigen by washing with PBST, blocking was carried out with 250 µL of 2% BSA-PBS for 1 hour or more. After removing the 2% BSA-PBS, the prepared culture supernatant was added and allowed to stand for 1 hour at 37°C for antibody binding. After washing, 50 mM PBS (pH 7.4) or 50 mM PBS (pH 5.5) was added and incubated by standing for 30 minutes at 37°C. After washing, detection was carried out with an HRP-conjugated anti-M13 antibody (Amersham Pharmacia Biotech) diluted with 2% BSA-PBS and TMB single solution (Zymed), followed by the addition of sulfuric acid to stop the reaction, and the measurement of absorbance at 450 nm.

**[0304]** However, no clones exhibiting potent pH-dependent binding ability were obtained by this panning using the antigen immobilized on the magnetic beads. Clones that were found to show weak pH-dependent binding ability were subjected to sequence analysis using specific primers. The positions in these clones where histidine was present at a high rate are shown in Table 3.

[Table 3] Positions of Histidine Substitution Detected Using Phage Library (Magnetic Bead Panning)

H50, H58, H61, H62, H63, H64, H65, H102  
L24, L27, L28, L32, L53, L56, L90, L92, L94

#### Acquisition of pH-Dependently Binding scFv from Library by Column Panning

**[0305]** No clones having strong pH-dependent binding ability were obtained by typical panning using the magnetic bead-immobilized antigen. This may be due to the following reasons. In the panning using an antigen immobilized on magnetic beads or a plate, all phages dissociated from the magnetic beads or plate under acidic conditions are collected. Thus, phage clones having weak pH dependency recovered together reduce the likelihood that clones having strong pH dependency are included in the finally concentrated clones.

**[0306]** Therefore, panning using a column immobilized with an antigen was examined as a more stringent panning method (Fig. 5). There have been no previous reports on the acquisition of clones having pH-dependent binding ability by using panning with an antigen-immobilized column. In the panning using an antigen-immobilized column, when phages that have been bound under neutral conditions are eluted under acidic conditions, clones having weak pH dependency rebound to the antigen within the column and are thereby less eluted, allowing strongly pH-dependent clones that less rebound within the column to be selectively eluted from the column. In addition, although "all" phages that have dissociated under acidic conditions are recovered in the panning using the antigen immobilized on magnetic beads or a plate, the panning using a column immobilized with the antigen enables selective recovery of phages having strong pH-dependent binding ability by allowing an acidic buffer to flow through the column to begin the elution and recovering only "appropriate fractions".

**[0307]** First, a column to which the antigen SR344 was immobilized was prepared. 200 µL of Streptavidin Sepharose (GE Healthcare) was washed with 1 mL of PBS, suspended in 500 µL of PBS, and contacted with 400 pmol of biotin-labeled SR344 for 1 hour at room temperature. Subsequently, an empty column (Amersham Pharmacia Biotech) was filled with the above sepharose and washed with about 3 mL of PBS. The above-mentioned PEG-precipitated library

phages were diluted to 1/25 with 0.5% BSA-PBS (pH 7.4), passed through a 0.45 nm filter, and then added to the column. After washing with about 6 mL of PBS (pH 7.4), 50 mM MES-NaCl (pH 5.5) was allowed to flow through the column to elute antibodies that dissociate under low pH. The appropriate eluted fractions were collected, and the recovered phage solution was added to 10 mL of logarithmic-growth-phase XL1-Blue (OD<sub>600</sub> of 0.4 to 0.5) and allowed to stand for 30 minutes at 37°C.

**[0308]** The infected *E. coli* were plated onto a 225 mm x 225 mm plate containing 2YT, 100 µg/mL ampicillin, and 2% glucose. These *E. coli* were used to begin additional phage culture in the same manner as described above and repeat the panning 6 times.

#### Evaluation by Phage ELISA

**[0309]** The resulting phages were evaluated by phage ELISA. Clones that were found to have strong pH dependency were subjected to sequence analysis using specific primers. As a result, several clones showing strong pH-dependent binding as compared to WT were obtained. As shown in Fig. 6, clone CL5 (H chain: CLH5, L chain: CLL5) (CLH5: amino acid sequence of SEQ ID NO: 5, CLL5: amino acid sequence of SEQ ID NO: 8) was found to exhibit particularly strong pH-dependent binding as compared to WT. It was thus confirmed that antibodies exhibiting strong pH-dependent binding, while being unable to be obtained by typical panning using the antigen immobilized onto magnetic beads, can be obtained by panning using a column immobilized with the antigen. Therefore, panning using an antigen-immobilized column was found to be a highly effective method for obtaining pH-dependently binding antibodies from a library. The amino acid sequences of the clones showing pH-dependent binding were analyzed, and the positions where histidine was present at a high probability in the concentrated clones are shown in Table 4.

[Table 4] Positions of Histidine Substitution found by Phage Library (Column Panning)

H31, H50, H58, H62, H63, H65, H100b, H102

L24, L27, L28, L32, L53, L56, L90, L92, L94

#### [Example 4] Expression and Purification of Histidine-Modified Humanized IL-6 Receptor Antibody

##### Production, Expression and Purification of Expression Vector of Histidine-Modified Humanized IL-6 Receptor Antibody

**[0310]** In order to convert clones showing strong pH dependency in phage ELISA to IgG, VH and VL were respectively amplified by PCR, digested with XhoI/NheI and EcoRI, and inserted to a mammalian cell expression vector. The nucleotide sequence of each DNA fragment was determined by a method known to persons skilled in the art. CLH5/L73, in which CLH5 was used for the H chain and L73 obtained in Example 2 was used for the L chain, was expressed and purified as IgG. Expression and purification were carried out by the method described in Example 1.

**[0311]** Antibody having even higher pH dependency was produced by combining mutation sites. Based on the locations where His was concentrated in the phage library as well as the structural information and the like, H32, H58, H62 and H102 in H3pl which was obtained as an H chain in Example 2 were substituted with histidine, and H95 and H99 were further substituted with valine and isoleucine, respectively, to produce H170 (SEQ ID NO: 4). The variant production was carried out using the method described in Example 1. In addition, L82 (SEQ ID NO: 7) was produced by substituting the 28th histidine of L73, which was produced as an L chain in Example 2, with aspartic acid. The variant production was carried out using the method described in Example 1. H170/L82, in which H170 was used for the H chain and L82 was used for the L chain, was expressed and purified as IgG using the method described in Example 1.

#### [Example 5] Evaluation of IL-6R-Neutralizing Activity of pH-Dependent Binding Antibody

##### Evaluation of Human IL-6 Receptor-Neutralizing Activity of Clones Converted to IgG

**[0312]** The IL-6 receptor-neutralizing activity was evaluated for four antibodies: humanized PM1 antibody (WT) and H3pl/L73, CLH5/L73 and H170/L82 produced in Examples 2 and 4.

**[0313]** More specifically, the IL-6 receptor-neutralizing activity was evaluated using BaF3/gp130 exhibiting IL-6/IL-6 receptor-dependent growth. BaF3/gp130 was washed three times with RPMI1640 medium containing 10% FBS, then suspended at  $5 \times 10^4$  cells/mL in RPMI1640 medium containing 60 ng/mL of human interleukin-6 (Toray), 60 ng/mL of recombinant soluble human IL-6 receptor (SR344) and 10% FBS. 50 µL of the suspension was dispensed into each of the wells of a 96-well plate (Corning). Next, the purified antibody was diluted with RPMI1640 containing 10% FBS, and 50 µL of the antibody was mixed into each well. After culturing for 3 days at 37°C and 5% CO<sub>2</sub>, WST-8 reagent (Cell Counting Kit-8, Dojindo Laboratories) diluted two-fold with PBS was added at 20 µL/well, and then immediately measured



for absorbance at 450 nm (reference wavelength: 620 nm) using the Sunrise Classic (Tecan). After culturing for 2 hours, absorbance at 450 nm was measured again (reference wavelength: 620 nm). The IL-6 receptor-neutralizing activity was evaluated based on the change in absorbance after 2 hours. As a result, as shown in Fig. 7, H3pl/L73, CLH5/L73 and H170/L82 were shown to have equivalent biological neutralization activity in comparison with the humanized PM1 antibody (WT).

#### [Example 6] Biacore Analysis of pH-Dependently Binding Antibody

##### Analysis of Binding of pH-Dependently Binding Clones to Soluble IL-6 Receptor

**[0314]** Kinetic analyses of antigen-antibody reactions at pH 5.8 and pH 7.4 were carried out using Biacore T100 (GE Healthcare) on the four antibodies: humanized PM1 antibody (WT) and H3pl/L73, CLH5/L73, and H170/L82 produced in Examples 2 and 4 (buffer: 10 mM MES (pH 7.4 or pH 5.8), 150 mM NaCl, and 0.05% Tween 20). Various antibodies were bound onto a sensor chip immobilized with recomb-protein A/G (pierce) by amine coupling. SR344 adjusted to concentrations of 9.8 to 400 nM was injected to the chip as an analyte. Association and dissociation of the pH-dependent binding clones to SR344 was observed in real time (Figs. 8 and 9). All the measurements were carried out at 37°C. Association rate constants  $k_a$  (1/Ms) and dissociation rate constants  $k_d$  (1/s) were calculated using Biacore T100 Evaluation Software (GE Healthcare), and dissociation constants KD (M) were calculated on the basis of those values (Table 5). Moreover, the ratio of the affinities at pH 5.8 and pH 7.4 were calculated for each clone to evaluate pH-dependent binding. All the measurements were carried out at 37°C.

**[0315]** As a result of calculating the affinity ratio between pH 5.8 and pH 7.4 for each clone, the pH-dependent binding (affinity) of H3pl/L73, H170/L82 and CLH5/L73 to SR344 was 41-fold, 394-fold and 66-fold, respectively, each showing the pH-dependent binding more than 15 times higher than WT.

**[0316]** Anti-IL-6 receptor antibodies that strongly bind to the antigen at the plasma pH of 7.4 but weakly bind to the antigen at the intraendosomal pH of 5.5 to 6.0 have not been reported yet. In this study, antibodies were obtained that retain the biological neutralization activity equivalent to the WT humanized IL-6 receptor antibody and the affinity at pH 7.4, but exhibit the affinity at pH 5.8 that has been specifically lowered more than 10 times.

[Table 5] Comparison of Binding of pH-Dependently Binding Clones Directed Against SR344 to Soluble IL-6 Receptor

		pH7.4			pH5.8			
	$k_a$ (1/Ms)	$k_d$ (1/s)	KD(M)	$k_a$ (1/Ms)	$k_d$ (1/s)	KD(M)	KD(pH5.8) /KD(pH7.4)	
WT	5.1E+05	1.0E-03	2.1E-09	7.6E+05	3.8E-03	5.0E-09	<b>2.4</b>	
H3pl/L73	5.4E+05	7.4E-04	1.4E-09	1.7E+05	9.7E-03	5.7E-08	<b>41.3</b>	
H170/L82	6.8E+05	1.1E-03	1.6E-09	2.6E+04	1.7E-02	6.4E-07	<b>393.5</b>	
CLH5/L73	7.1E+05	7.9E-04	1.1E-09	3.8E+05	2.8E-02	7.4E-08	<b>66.1</b>	

##### Analysis of Binding of pH-Dependently Binding Clones to Membrane IL-6 Receptor

**[0317]** Antigen-antibody reactions to membrane IL-6 receptor at pH 5.8 and pH 7.4 were observed for the above produced pH-dependent binding clones, using Biacore T100 (GE Healthcare). The binding to membrane IL-6 receptor was evaluated by evaluating the binding to the IL-6 receptor immobilized onto a sensor chip. SR344 was biotinylated according to a method known to persons skilled in the art, and the biotinylated SR344 was immobilized on the sensor chip via streptavidin by utilizing the affinity between streptavidin and biotin. All the measurements were carried out at 37°C, and the mobile phase buffer contained 10 mM MES (pH 5.8), 150 mM NaCl and 0.05% Tween 20. The pH-dependent binding clones were injected therein under the condition of pH 7.4 and allowed to bind to SR344 (injection sample buffer: 10 mM MES (pH 7.4), 150 mM NaCl, 0.05% Tween 20), and the pH-dependent dissociation of each clone at the mobile phase pH of 5.8 was observed (Fig. 10).

**[0318]** The dissociation rate ( $k_d$ (1/s)) at pH 5.8 was calculated using Biacore T100 Evaluation Software (GE Healthcare) by fitting only the dissociation phase at pH 5.8, where 0.5 µg/mL of the sample was bound in 10 mM MES (pH 7.4), 150 mM NaCl, and 0.05% Tween 20, and dissociated in 10 mM MES (pH 5.8), 150 mM NaCl, and 0.05% Tween 20. Similarly, the dissociation rate ( $k_d$ (1/s)) at pH 7.4 was calculated using Biacore T100 Evaluation Software (GE Healthcare) by fitting only the dissociation phase at pH 7.4, where 0.5 µg/mL of the sample was bound in 10 mM MES (pH 7.4), 150 mM NaCl, and 0.05% Tween 20, and dissociated in 10 mM MES (pH 7.4), 150 mM NaCl, and 0.05% Tween 20. The pH-dependent dissociation rate constant of each clone is shown in Table 6.

[Table 6] Comparison of Rate Constant of Dissociation of pH-Dependent Binding Clones directed against SR344 from Membrane IL-6 Receptor

	kd(1/s)		kd ratio
	pH7.4	pH5.8	pH5.8/pH7.4
WT	4.B4E-04	7.15E-04	1.5
H3pl/L7 <sub>3</sub>	3.44E-04	3.78E-03	11.0
H170/L82	7.70E-04	1.44E-03	1.9
CLH5/L73	1.04E-03	5.67E-03	5.5

[0319] The highest pH dependency of the dissociation ratio was observed in H3pl/L73 followed by CLH5/L73 and H170/L82 in descending order, and each clone demonstrated higher pH-dependent dissociation from the membrane IL-6 receptor than WT. However, the rank of pH-dependent association/dissociation was different between the soluble IL-6 receptor and membrane IL-6 receptor. It was revealed that H170/L82, which exhibited the highest pH-dependent binding in the analysis of binding to soluble IL-6 receptor, showed the lowest pH-dependent binding in the analysis of binding to the membrane IL-6 receptor. In general, it is known that while IgG molecules monovalently bind to a soluble antigen (affinity), they divalently bind to membrane antigens (avidity). It is suggested that this difference in the binding mode between soluble antigens and membrane antigens influenced the pH-dependent binding of H170/L82.

#### [Example 7] Confirmation of Multiple Binding to Antigen by pH-Dependent Binding Antibody

[0320] As described in Example 2, pH-dependent binding antibodies may be able to bind to antigens multiple times. Specifically, a pH-dependent binding antibody that has bound to an antigen is non-specifically taken up into endosomes, but dissociated from the soluble antigen under the intraendosomal acidic conditions. The antibody binds to FcRn and thereby returns to the plasma. Since the antibody that has returned to the plasma is not bound to antigen, it is able to bind to a new antigen again. The repetition of this process enables pH-dependent binding antibodies to bind to antigens multiple times. However, for IgG antibodies that do not have pH-dependent binding ability, not all antigens are dissociated from the antibodies under the intraendosomal acidic conditions. Thus, such antibodies that have been returned to the plasma by FcRn remain bound to antigen, and therefore cannot bind to new antigens. Consequently, in nearly all cases, each single molecule of IgG antibodies is able to neutralize only two antigens (in the case of divalent binding).

[0321] Therefore, it was evaluated whether the three pH-dependently binding antibodies (H3pl/L73, CLH5/L73, and H170/L82) constructed in Examples 2 and 4 were able to bind to the antigen SR344 multiple times as compared to the humanized PM1 antibody (wild type, WT).

[0322] Biacore (GE Healthcare) was used to evaluate that the antibodies binding at pH 7.4 and dissociating at pH 5.8 were able to bind to the antigen multiple times. The antibody to be evaluated was bound to a recomb-protein A/G (Pierce)-immobilized sensor chip by the amine coupling method, and a mobile phase of pH 7.4 was allowed to flow (step 1). SR344 solution adjusted to pH 7.4 was then allowed to flow as an analyte to bind SR344 to the antibody at pH 7.4 (step 2). This binding at pH 7.4 mimics the antigen binding in plasma. Subsequently, buffer adjusted to pH 5.8 alone (not containing SR344) was added as an analyte to expose the antigen bound to the antibody to acidic conditions (step 3). This dissociation at pH 5.8 mimics the binding state of antigen-antibody complexes in endosomes. Subsequently, step 2 was repeated. This mimics the rebinding of antibody that has been returned to plasma by FcRn to a new antigen. Subsequently, step 2 was repeated to expose the antibody-antigen complex to acidic conditions. Repeating "step 2 to step 3" multiple times at 37°C as described above can mimic the in vivo state in which antibodies are repeatedly taken up from the plasma into endosomes by pinocytosis and returned to the plasma by FcRn (Nat. Rev. Immunol. 2007 Sep; 7(9): 715-25).

[0323] The produced pH-dependent binding clones described above were analyzed using Biacore T100 (GE Healthcare) for their ability to bind to the antigen SR344 multiple times at pH 5.8 and pH 7.4. More specifically, the analysis was carried out as follow. All the measurements were carried out at 37°C. First, the sample antibodies described above were bound onto a recomb-protein A/G (Pierce)-immobilized sensor chip by amine-coupling, where the mobile phase buffer was 10 mM MES (pH 5.8), 150 mM NaCl, and 0.05% Tween 20 (step 1). SR344 adjusted to a concentration of about 40 nM was injected as an analyte for 3 minutes at pH 7.4 and allowed to bind (the buffer for injected SR344 was 10 mM MES (pH 7.4), 150 mM NaCl, and 0.05% Tween 20) (step 2). Subsequently, the injection of SR344 was discontinued and a mobile phase of pH 5.8 was allowed to flow for about 70 seconds to expose the antibody/SR344 complex under acidic conditions (step 3). Ten sets of this binding (step 2)/acidity exposure (step 3) process were continuously repeated to observe the sensorgram in real-time, which is shown in Fig. 11. WT showed less dissociation of SR344

during the acidic exposure in step 3, and consequently the proportion of antibody capable of binding to new antigens in the subsequent step 2 was extremely low. In contrast, it was found that the pH-dependent binding clones, particularly H170/L82 and CLH5/L73, demonstrated so strong dissociation during the acidic exposure in step 3 that most of the bound SR344 was dissociated, and therefore nearly all antibodies were able to bind to new antigens in the subsequent step 2. In the 10-set repetition of the binding (step 2) and acidic exposure (step 3), almost all H170/L82 and CLH5/L73 antibodies were able to bind to new antigens in each set.

**[0324]** The obtained sensorgrams were used to calculate the binding amount of SR344 in each set for each sample using Biacore T100 Evaluation Software (GE Healthcare). The integrated values in the time course of the 10 sets are shown in Fig. 12. The integrated RU values obtained at the 10th set are equivalent to the total amount of antigens bound during the ten cycles. The pH-dependent binding clones, particularly H170/L82 and CLH5/L73, showed the largest total amounts of bound antigens in comparison with WT, and were demonstrated to be able to repeatedly bind to roughly four times the amount of antigens bound by WT. Accordingly, it was revealed that by conferring pH-dependent binding ability to WT, such antibodies can repeatedly bind to antigens and thereby neutralize multiple antigens.

#### **[Example 8] PK/PD Test of pH-Dependently-Binding Antibody Using Human IL-6 Receptor Transgenic Mice**

**[0325]** IL-6 receptors are present in the body in both soluble IL-6 receptor form and membrane-type IL-6 receptor form (Nat. Clin. Pract. Rheumatol. 2006 Nov; 2(11): 619-26). Anti-IL-6 receptor antibodies bind to soluble IL-6 receptors and membrane-type IL-6 receptors and neutralize their biological action. It is believed that an anti-IL-6 receptor antibody binds to a membrane-type IL-6 receptor, is subsequently taken up into an endosome within a cell by internalization while the antibody is kept bound to the membrane-type IL-6 receptor, and then moves to a lysosome while still kept bound to the membrane-type IL-6 receptor where it is degraded by lysosome together with the membrane-type IL-6 receptor. If H3pl/L73, CLH5/L73, and H170/L82, which are the pH-dependent-binding IL-6 receptor antibodies evaluated in Example 6, are able to return to plasma via FcRn as a result of dissociation under acidic conditions within endosomes, the antibodies that have returned to the plasma can bind to antigens again. This enables neutralization of multiple membrane-type IL-6 receptors with a single antibody molecule. Whether or not the return to the plasma via FcRn as a result of dissociation under acidic conditions within endosomes is achieved with the constructed pH-dependent-binding anti-IL-6 receptor antibodies can be determined by evaluating whether the pharmacokinetics of these antibodies are improved as compared with that of WT.

**[0326]** Thus, the pharmacokinetics in human-IL-6-receptor transgenic mice (hIL-6R tg mice, Proc. Natl. Acad. Sci. USA 1995 May 23; 92(11): 4862-6) was evaluated for the four types of antibodies, that is, humanized PM1 antibody (wild type: WT) and H3pl/L73, CLH5/L73, and H170/L82 constructed in Examples 2 and 4. WT, H3pl/L73, CLH5/L73, or H170/L82 was administered by single-dose intravenous administration to hIL-6R tg mice at 25 mg/kg, and blood samples were collected, before administration and over time. Collected blood was immediately centrifuged for 15 minutes at 15,000 rpm and 4°C to obtain plasma. Separated plasma was stored in a freezer set to -20°C or lower until measurements were carried out.

**[0327]** The measurement of concentration in mouse plasma was carried out by ELISA. Samples for calibration curve were prepared at plasma concentrations of 6.4, 3.2, 1.6, 0.8, 0.4, 0.2 and 0.1 µg/mL. The calibration curve samples and mouse plasma measurement samples were dispensed into an immunoplate (Nunc-Immuno Plate, MaxiSorp (Nalge Nunc International)) immobilized with anti-human IgG (γ-chain specific) F(ab')<sub>2</sub> (Sigma), and allowed to stand undisturbed for one hour at room temperature. Goat anti-human IgG-BIOT (Southern Biotechnology Associates) and streptavidin-alkaline phosphatase conjugate (Roche Diagnostics) were sequentially allowed to react, and a chromogenic reaction was carried out by using BluePhos Microwell Phosphatase Substrates System (Kirkegaard & Perry Laboratories) as substrate. Absorbance at 650 nm was measured with a microplate reader. The concentrations in mouse plasma were calculated from the absorbance of the calibration curve using the analytical software SOFTmax PRO (Molecular Devices). Time courses of plasma concentrations of WT as well as H3pl/L73, CLH5/L73, and H170/L82 are shown in FIG. 13.

**[0328]** The pharmacokinetics was improved for all of H3pl/L73, CLH5/L73, and H170/L82 as compared with WT. In particular, the pharmacokinetics of H3pl/L73 and CLH5/L73 were improved considerably. A wild type anti-IL-6 receptor antibody (WT) bound to membrane-type IL-6 receptor is taken up into an endosome within a cell by internalization, moves to a lysosome while the antibody is kept bound to the antigen, and then degraded; therefore, it has a short residence time in the plasma. In contrast, since the pharmacokinetics of the pH-dependent-binding anti-IL-6 receptor antibodies were improved considerably, the pH-dependent-binding anti-IL-6 receptor antibodies were thought to return to the plasma again via FcRn as a result of dissociation from the antigen, membrane-type IL-6 receptor, under acidic conditions within endosomes.

**[0329]** Although the pharmacokinetics was improved for all of H3pl/L73, CLH5/L73, and H170/L82 as compared with WT, the effect of prolonging plasma persistence time of H170/L82 was weaker than that of H3pl/L73 and CLH5/L73. Since IgG molecules are thought to normally bind divalently to membrane-bound antigen, it is thought that anti-IL-6 receptor antibodies also bind divalently (avidity) to membrane-type IL-6 receptors and then are internalized. As indicated

in Example 6, the analysis using Biacore revealed that H170/L82 rapidly dissociated from the IL-6 receptor at pH 5.8 when binding to soluble IL-6 receptor (FIG. 9), but the dissociation rate thereof from the IL-6 receptor at pH 5.8 when binding to membrane-type IL-6 receptor was extremely slow (FIG. 10). From this result, the reason for the weak effect of prolonging residence time in plasma of H170/L82 is thought to be that the antibody was unable to adequately dissociate within endosomes after having been internalized due to its slow dissociation at pH 5.8 when binding to membrane-type IL-6 receptor. Namely, as for the case relating to membrane antigen, it was determined that in order for a single IgG molecule to neutralize multiple membrane antigens, the pH dependency of dissociation from divalent binding (avidity) is more important than the pH dependency of monovalent binding (affinity).

#### [Example 9] PK/PD Test of pH-Dependently-Binding Antibody Using Cynomolgus Monkeys

**[0330]** Since the pharmacokinetics of the pH-dependently-binding anti-IL-6 receptor antibodies were improved considerably in Example 8, the pH-dependently-binding anti-IL-6 receptor antibodies were thought to return to plasma via FcRn as a result of dissociation from the antigen, membrane-type IL-6 receptor, under acidic conditions within endosomes. If antibodies that have returned to the plasma can bind to membrane-type IL-6 receptors again, neutralization of an antigen, the membrane-type IL-6 receptor, by pH-dependently-binding anti-IL-6 receptor antibodies is thought to persist longer than that by the wild-type anti-IL-6 receptor antibody, at the same dosage. In addition, since the soluble IL-6 receptor is also present among IL-6 receptors, the duration of neutralization is thought to be longer for the same dosage with respect to the soluble IL-6 receptor as well.

**[0331]** The pharmacokinetics in cynomolgus monkeys was evaluated for WT and H3pl/L73. WT or H3pl/L73 was administered to cynomolgus monkeys by single-dose intravenous administration at 1 mg/kg, and blood samples were collected, before administration and over time. Collected blood was immediately centrifuged for 15 minutes at 15,000 rpm and 4°C to obtain plasma. The separated plasma was stored in a freezer set to -20°C or lower until measurements were carried out.

**[0332]** The measurement of concentration in cynomolgus monkey plasma was carried out by ELISA. First, anti-human IgG ( $\gamma$ -chain specific) F(ab')<sub>2</sub> fragment of antibody (Sigma) was dispensed into a Nunc-ImmunoPlate MaxiSorp (Nalge Nunc International) and allowed to stand undisturbed overnight at 4°C to prepare plates immobilized with anti-human IgG. Calibration curve samples having plasma concentrations of 3.2, 1.6, 0.8, 0.4, 0.2, 0.1 and 0.05  $\mu$ g/mL and cynomolgus monkey plasma measurement samples diluted 100-fold or more were prepared; 200  $\mu$ L of 20 ng/mL cynomolgus monkey IL-6R was added to 100  $\mu$ L of the calibration curve samples and plasma measurement samples; and then, they were allowed to stand undisturbed for one hour at room temperature. Subsequently, the samples were dispensed into the anti-human IgG-immobilized plate and allowed to stand undisturbed for another one hour at room temperature. Biotinylated Anti-Human IL-6R Antibody (R&D) was allowed to react for one hour at room temperature, and then Streptavidin-PolyHRP80 (Stereospecific Detection Technologies) was allowed to react for one hour. A chromogenic reaction was carried out by using TMP One Component HRP Microwell Substrate (BioFX Laboratories) as substrate, then, the reaction was stopped with 1N sulfuric acid (Showa Chemical) and the absorbance at 450 nm was measured with a microplate reader. The concentrations in cynomolgus monkey plasma were calculated from the absorbance of the calibration curve using the analytical software SOFTmax PRO (Molecular Devices). The time courses of plasma concentrations of WT and H3pl/L73 after the intravenous administration are shown in FIG. 14. As a result, the pharmacokinetics of H3pl/L73 was improved considerably in comparison with WT in cynomolgus monkeys in the same manner as in human IL-6 receptor transgenic mice. Since the pharmacokinetics of a pH-dependent-binding anti-IL-6 receptor antibody, H3pl/L73, was improved considerably, H3pl/L73 was thought to return to the plasma via FcRn as a result of dissociation from the antigen, membrane-type IL-6 receptor, under acidic conditions within endosomes.

**[0333]** In order to evaluate the degree to which cynomolgus monkey membrane-type IL-6 receptor is neutralized by the intravenous administration of WT and H3pl/L73, the effects of simple antibodies on plasma C-reactive protein (CRP) induced by cynomolgus monkey IL-6 were studied. Since CRP is secreted when IL-6 binds to membrane-type IL-6 receptors, CRP serves as an indicator of neutralization of membrane-type IL-6 receptors. Cynomolgus monkey IL-6 (cyno.IL-6 prepared in Example 1) containing 1% inactivated cynomolgus monkey plasma was administered subcutaneously into lower backs of the animals daily at 5  $\mu$ g/kg from day 3 to day 10 after the administration of WT or H3pl/L73. Blood samples were collected from the saphenous vein immediately before the start of cynomolgus monkey IL-6 administration (day 3) and after the administration at 24-hour intervals (day 4 to day 11), then were separated into plasma. The CRP concentrations of individual animals were measured with Cias R CRP (Kanto Chemical) using an automated analyzer (TBA-120FR, Toshiba Medical Systems). The time courses of CRP concentration upon induction with cynomolgus IL-6 with respect to WT and H3pl/L73 are shown in FIG. 15. As a result, the duration of CRP suppression was prolonged considerably by H3pl/L73 in comparison with WT. On the basis of this finding, a pH-dependent-binding anti-IL-6 receptor antibody, H3pl/L73, was thought to return to the plasma via FcRn as a result of dissociation from its antigen, membrane-type IL-6 receptor, under acidic conditions within endosomes; and neutralize the membrane-type IL-6 receptor by re-binding thereto; and thereby suppress production of CRP for a longer period of time than WT. In other words,

H3pl/L73 was shown to be able to bind to and neutralize the membrane-type IL-6 receptor more than once, as a single antibody molecule. Since the duration of suppression of CRP production by H3pl/L73 is prolonged in comparison to that by WT, the duration of time where an antigen, the membrane-type IL-6 receptor, is bound by antibodies was indicated to be prolonged for H3pl/L73 than WT.

**[0334]** In order to evaluate the degree to which cynomolgus monkey soluble IL-6 receptor is neutralized by the intravenous administration of WT and H3pl/L73, the concentration of unbound cynomolgus monkey soluble IL-6 receptor in cynomolgus monkey plasma was measured. All IgG-type antibodies (cynomolgus monkey IgG, anti-human IL-6 receptor antibody, and a complex of anti-human-IL-6 receptor antibody and cynomolgus monkey soluble IL-6 receptor) present in the plasma were adsorbed to Protein A by adding 30  $\mu$ L of cynomolgus monkey plasma to an appropriate amount of rProtein A Sepharose Fast Flow (GE Healthcare) resin dried in a 0.22  $\mu$ m filter cup (Millipore). After spinning down with a high-speed centrifuge, the solution that passed through (hereinafter referred to as "pass solution") was recovered. Since the pass solution does not contain the complex of anti-human IL-6 receptor antibody and Cynomolgus monkey soluble IL-6 receptor which is bound to protein A, the concentration of unbound soluble IL-6 receptor can be measured by measuring the concentration of cynomolgus monkey soluble IL-6 receptor in the pass solution. Monoclonal Anti-human IL-6R Antibody (R&D) that was ruthenium-labeled with Sulfo-Tag NHS Ester (Meso Scale Discovery) and Biotinylated Anti-human IL-6R Antibody (R&D) were mixed with cynomolgus monkey IL-6 receptor calibration curve samples adjusted to concentrations of 4000, 2000, 1000, 500, 250, 125, and 62.5 pg/mL and the plasma samples treated with Protein A as described above. The mixtures were allowed to react for one hour at room temperature. Subsequently, the mixtures were dispensed into an SA-Coated Standard MA2400 96-well plate (Meso Scale Discovery). After allowing to react for another one hour and washing, Read Buffer T (x4) (Meso Scale Discovery) was dispensed. Immediately thereafter, the measurement with Sector Imager 2400 (Meso Scale Discovery) was conducted. The concentrations of cynomolgus monkey IL-6 receptor were calculated from the response of the calibration curve by using the analytical software, SOFTmax PRO (Molecular Devices). The time courses of concentrations of unbound cynomolgus monkey soluble IL-6 receptor for WT and H3pl/L73 are shown in FIG. 16. As a result, the duration of neutralization of cynomolgus monkey soluble IL-6 receptor by H3pl/L73 was considerably prolonged as compared to that by WT. On the basis of this finding, the pH-dependent-binding anti-IL-6 receptor antibody H3pl/L73 was thought to dissociate from its antigen, soluble IL-6 receptor, under acidic conditions in endosomes; and return to the plasma via FcRn; and bind to and neutralize the soluble IL-6 receptor again. Since the duration of suppression of unbound cynomolgus monkey soluble IL-6 receptor by H3pl/L73 is prolonged in comparison to that by WT, the duration of time where an antigen, the soluble IL-6 receptor, is bound by antibodies was indicated to be prolonged for H3pl/L73 than WT.

**[0335]** From these findings, the time until the antibody disappears from the plasma as well as the time where soluble and membrane-type IL-6 receptors are bound by the antibody in the body were found to be considerably elongated for the pH-dependent-binding anti-IL-6 receptor antibodies that were made to bind strongly to the antigen at pH 7.4, which is the pH in the plasma, but bind weakly to the antigen at pH 5.8, which is the pH within endosomes, as compared to the wild-type anti-IL-6 receptor antibody. This makes it possible to reduce the dosage and frequency of administration to patients, and in consequence, the total administration dosage. Therefore, the pH-dependent-binding anti-IL-6 receptor antibody is thought to be particularly advantageous as a pharmaceutical for use as an IL-6 antagonist.

[Example 10] Improvement of pH-Dependent Binding to Membrane-Type IL-6 Receptor by Optimization of Variable Region

#### Optimization of Variable Regions H3pl/L73 and CLH5/L82

**[0336]** Antibodies having pH-dependent binding abilities were shown to demonstrate superior effects in Example 9. Therefore, to further improve the pH-dependent binding abilities, mutations were introduced into the CDR sequence of CLH5 obtained in Example 3 to construct VH1-IgG1 (SEQ ID NO: 21) and VH2-IgG1 (SEQ ID NO: 22). In addition, mutations were introduced into the framework sequence and CDR sequence of H3pl to construct the modified H chains VH3-IgG1 (SEQ ID NO: 23) and VH4-IgG1 (SEQ ID NO: 24). Mutations were introduced into the CDR sequences of L73 and L82 to construct the modified L chains VL1-CK (SEQ ID NO: 25), VL2-CK (SEQ ID NO: 26), and VL3-CK (SEQ ID NO: 27). More specifically, the mutants were constructed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the method described in the appended instructions, and the resulting plasmid fragments were inserted into an mammalian cell expression vector to construct the desired H chain expression vectors and L chain expression vectors. The nucleotide sequences of the resulting expression vectors were determined by methods known to persons with ordinary skill in the art.

**[0337]** The antibody having VH2-IgG1 (SEQ ID NO: 22) as H chain and VL2-CK (SEQ ID NO: 26) as L chain was denoted as Fv1-IgG1, the antibody having VH1-IgG1 (SEQ ID NO: 21) as H chain and L82 as L chain was denoted as Fv2-IgG1, the antibody having VH4-IgG1 (SEQ ID NO: 24) as H chain and VL1-CK (SEQ ID NO: 25) as L chain was denoted as Fv3-IgG1, and the antibody having VH3-IgG1 (SEQ ID NO: 23) as H chain and VL3-CK (SEQ ID NO: 27)



as L chain was denoted as Fv4-IgG1. Of these, Fv2-IgG1 and Fv4-IgG1 were expressed and purified. The expression and purification were carried out by the method described in Example 1.

#### Analysis of Binding of pH-Dependent-Binding Clones to Soluble IL-6 Receptor

**[0338]** Kinetic analyses of antigen-antibody reactions at pH 7.4 were carried out on the four types of antibodies, i.e., the humanized PM1 antibody (wild type: WT), and WT, H3pl/L73-IgG1, Fv2-IgG1, and Fv4-IgG1 constructed in Examples 2 and 10, by using Biacore T100 (GE Healthcare) (buffer: 10 mM MES (pH 7.4), 150 mM NaCl, 0.05% Tween 20). Each antibody was bound on a sensor chip on which an anti-IgG  $\gamma$  chain specific F(ab)<sub>2</sub> (Pierce) was immobilized by amine coupling, and then, SR344 adjusted to a concentration of 9.8 to 40 nM was injected thereto as an analyte. The association to and dissociation from SR344 were observed on a real-time basis for the pH-dependent-binding clones. All the measurements were carried out at 37°C. Association rate constants  $k_a$  (1/Ms) and dissociation rate constants  $k_d$  (1/s) were calculated using Biacore T100 Evaluation Software (GE Healthcare), and dissociation constants  $K_D$  (M) were calculated on the basis of those values (Table 7).

[Table 7] Comparison of Dissociation Rate Constants of pH-Dependent-Binding Clones from Soluble IL-6 Receptor, SR344

Sample	$k_a$ (1/Ms)	$k_d$ (1/s)	$K_D$ (M)
WT	4.0E+05	1.1E-03	2.7E-09
H3pl/L73	4.1E+05	5.9E-04	1.4E-09
Fv2-IgG1	3.9E+05	7.7E-04	2.0E-09
Fv4-IgG1	7.2E+05	1.0E-03	1.4E-09

**[0339]** As a result of calculating the affinity at pH 7.4 for each clone, the dissociation constants (affinity,  $K_D$  value) of WT, H3pl/L73-IgG1, Fv2-IgG1, and Fv4-IgG1 to SR344 were, respectively, 2.7 nM, 1.4 nM, 2.0 nM, and 1.4 nM, and they are nearly equivalent. Fv2-IgG1 and Fv4-IgG1 were demonstrated to have binding ability to the soluble IL-6 receptor that is equal to or greater than that of WT.

#### Analysis of Binding of pH-Dependent-Binding Clones to Membrane-Type IL-6 Receptor

**[0340]** Antigen-antibody reactions to membrane-type IL-6 receptor were observed at pH 5.8 and pH 7.4 for the four types of constructed clones, WT, H3pl/L73-IgG1, Fv2-IgG1, and Fv4-IgG1 by using Biacore T100 (GE Healthcare). Binding to the membrane-type IL-6 receptor was evaluated by evaluating binding to the IL-6 receptor immobilized on a sensor chip. SR344 was biotinylated in accordance with a method known among persons with ordinary skill in the art, and the biotinylated SR344 was immobilized on the sensor chip via streptavidin using the affinity between streptavidin and biotin. All the measurements were carried out at 37°C. The mobile phase buffer was 10 mM MES (pH 5.8), 150 mM IvaC1 and 0.05% Tween 20. The pH-dependent-binding clones were injected therein under conditions of pH 7.4 to allow them to bind to SR344 (injection sample buffer: 10 mM MES (pH 7.4), 150 mM NaCl, 0.05% Tween 20), then, pH-dependent dissociation of each clone was observed at the pH of the mobile phase of 5.8 (FIG. 17).

**[0341]** Sample concentrations were adjusted to 0.25  $\mu$ g/mL. Binding was carried out in 10 mM MES (pH 7.4), 150 mM NaCl, and 0.05% Tween 20. Dissociation was carried out in 10 mM MES (pH 5.8), 150 mM NaCl, and 0.05% Tween 20. For this case, the dissociation rate constants ( $k_d$ (1/s)) at pH 5.8 were calculated by fitting only the dissociation phase at pH 5.8 using Biacore T100 Evaluation Software (GE Healthcare). In a similar manner, sample concentrations were adjusted to 0.25  $\mu$ g/mL, binding was carried out in 10 mM MES (pH 7.4), 150 mM NaCl, and 0.05% Tween 20, dissociation was carried out in 10 mM MES (pH 7.4), 150 mM NaCl, and 0.05% Tween 20, and the dissociation rate constants ( $k_d$  (1/s)) at pH 7.4 were calculated by fitting only the dissociation phase at pH 7.4 using Biacore T100 Evaluation Software (GE Healthcare). The pH-dependent dissociation rate constants of each clone are shown in Table 8.

[Table 8] Comparison of Dissociation Rate Constants of pH-Dependent-Binding Clones from Membrane-Type IL-6 Receptor, SR344

Sample	pH7.4 $k_d$ (1/s)	pH5.8 $k_d$ (1/s)	pH Dependency $k_d(\text{pH}5.8)/k_d(\text{pH}7.4)$
WT	2.5E-04	2.5E-04	1.00
H3pl/L73	2.6E-04	6.7E-04	2.59
Fv2-IgG1	3.4E-04	2.4E-03	7.18

(continued)

Sample	pH7.4 kd (1/s)	pH5.8 kd (1/s)	pH Dependency kd(pH5.8)/kd(pH7.4)
Fv4-IgG1	4.7E-04	2.6E-03	5.56

[0342] As a result of calculating pH dependency for each clone, the pH dependencies of binding to the membrane-type IL-6 receptor of the four clones, WT, H3pl/L73-IgG1, Fv2-IgG1, and Fv4-IgG1 with respect to SR344 were 1.0-fold, 2.59-fold, 7.18-fold, and 5.56-fold, respectively. Fv2-IgG1 and Fv4-IgG1 demonstrated higher pH-dependency in dissociation from the membrane-type IL-6 receptor than H3pl/L73-IgG1.

[0343] On the basis of the above, Fv2-IgG1 and Fv4-IgG1 were shown to demonstrate stronger pH-dependent binding to the membrane-type IL-6 receptor than H3pl/L73-IgG1 while maintaining the affinity for the soluble IL-6 receptor equal to or stronger than that of WT.

[Example 11] PK/PD Test of pH-Dependent-Binding Antibodies with Optimized Variable Regions Using Human IL-6 Receptor Transgenic Mice

[0344] The pharmacokinetics of Fv2-IgG1 and Fv4-IgG1, as well as WT and H3pl/L73-IgG1 prepared and evaluated in Example 10 were evaluated using the human IL-6 receptor transgenic mice used in Example 8. WT, H3pl/L73-IgG1, Fv2-IgG1, or Fv4-IgG1 was administered by single-dose intravenous administration to hIL-6R tg mice at 25 mg/kg, and the concentration of each antibody in the plasma was measured in the same manner as in Example 8. The time courses of the plasma concentrations of WT, H3pl/L73-IgG1, Fv2-IgG1, and Fv4-IgG1 are shown in FIG. 18.

[0345] The pharmacokinetics of H3pl/L73-IgG1 improved in comparison with WT in the same manner as in Example 8, while the pharmacokinetics of Fv2-IgG1 and Fv4-IgG1 was further improved than H3pl/L73-IgG1. Measurement as to the unbound IL-6 receptor concentrations, as measured in cynomolgus monkeys in Example 9, was carried out in the hIL-6R tg mice in this test using the same method. As a result, prolongation of the duration of neutralization of the soluble IL-6 receptor was confirmed for Fv2-IgG1 and Fv4-IgG1 in comparison to that for H3pl/L73-IgG1 (data not shown). As indicated in Example 10, the pH-dependent binding to the membrane-type IL-6 receptor was improved for Fv2-IgG1 and Fv4-IgG1 as compared with H3pl/L73-IgG1. Therefore, it was indicated that further improvement in the pharmacokinetics and duration of neutralization of the soluble IL-6 receptor over those of H3pl/L73-IgG1 is possible by improving the pH-dependent binding to the membrane-type IL-6 receptor.

[Example 12] Improvement of pH-Dependent Binding to Membrane-Type IL-6 Receptor by Optimization of Constant Region

#### Optimization of Constant Regions of Fv4-IgG1

[0346] Generally, binding to membrane-bound antigens has been reported to vary depending on the constant region of the antibody (J. Immunol. Methods 1997 Jun 23; 205(1): 67-72). The constant regions of the pH-dependent-binding antibodies prepared above were of the IgG1 isotype. Therefore, a study was made for optimization of the constant region in order to improve the pH-dependent binding to the membrane-type IL-6 receptor.

[0347] A mutation was introduced into a naturally-occurring constant region, i.e., constant region IgG2 (SEQ ID NO: 28), to construct constant region IgG2ΔGK (SEQ ID NO: 29). Another mutation was introduced into the constant region IgG2ΔGK to construct constant region M58 (SEQ ID NO: 30). Mutations were further introduced into the constant regions IgG2 and M58 to construct constant regions M71 (SEQ ID NO: 31) and M73 (SEQ ID NO: 32).

[0348] VH3-IgG2ΔGK (SEQ ID NO: 33) was constructed by substituting the constant region of VH3-IgG1 prepared in Example 10 with IgG2ΔGK, VH3-M58 (SEQ ID NO: 34) was constructed by substituting the constant region with M58, and VH3-M73 (SEQ ID NO: 35) was constructed by substituting the constant region with M73. More specifically, expression vectors in which the constant region portion of VH3 used in Example 10 was substituted by a desired constant region by NheI/NotI digestion and ligation were constructed. The nucleotide sequences of the resulting expression vectors were determined using a method known among persons with ordinary skill in the art.

[0349] Expression and purification were carried out for the following: Fv4-IgG2 using VH3-IgG2ΔGK (SEQ ID NO: 33) for the H chain and VL3-CK (SEQ ID NO: 27) for the L chain; Fv4-M58 using VH3-M58 (SEQ ID NO: 34) for the H chain and VL3-CK (SEQ ID NO: 27) for the L chain; and Fv4-M73 using VH3-M73 (SEQ ID NO: 35) for the H chain and VL3-CK (SEQ ID NO: 27) for the L chain. Expression and purification were carried out using the method described in Example 1.

Analysis of Binding of Fv4 Having Optimized Constant Region to Soluble IL-6 Receptor

[0350] The association with and dissociation from SR344 were observed on a real-time basis using the same method as Example 10 for thus-prepared Fv4-IgG1, Fv4-IgG2, Fv4-M58, and Fv4-M73 as well as WT. The association rate constants  $k_a$  (1/Ms) and dissociation rate constants  $k_d$  (1/s) were calculated after analysis in the same manner, and then, dissociation constants  $K_D$  (M) were calculated on the basis of those values (Table 9).

[Table 9] Comparison of Dissociation Rate Constants of pH-Dependently-Binding Clones from Soluble IL-6 Receptor, SR344

Sample	$k_a$ (1/Ms)	$k_d$ (1/s)	$K_D$ (M)
Fv4-IgG1	7.2E+05	1.0E-03	1.4E-09
Fv4-IgG2	9.6E+05	1.2E-03	1.3E-09
Fv4-M58	8.3E+05	1.1E-03	1.4E-09
Fv4-M73	7.5E+05	1.0E-03	1.4E-09

[0351] As a result of calculating the affinity at pH 7.4 for each clone, the dissociation constants (affinity,  $K_D$  value) of Fv4-IgG1, Fv4-IgG2, Fv4-M58, and Fv4-M73 to SR344 were 1.4 nM, 1.3 nM, 1.4 nM, and 1.4 nM, respectively, and they are almost equivalent. This indicates that the binding ability of pH-dependent-binding clones to the soluble IL-6 receptor, SR344, does not change even after modifying the constant region. On the basis of this finding, the binding ability to the soluble IL-6 receptor was thought to not change for Fv1, Fv2, and Fv3 even if the constant region was similarly modified.

Analysis of Binding of Fv4 Having Optimized Constant Region to Membrane-Type IL-6 Receptor

[0352] Antigen-antibody reactions to the membrane-type IL-6 receptor at pH 5.8 and pH 7.4 were observed for thus-prepared Fv4-IgG1, Fv4-IgG2, Fv4-M58, and Fv4-M73 as well as WT, in the same manner as in Example 10 using Biacore T100 (GE Healthcare). The results obtained by injecting the pH-dependent-binding clones under the conditions of pH 7.4 to allow binding to SR344, and by observing the pH-dependent dissociation of each clone in the pH 5.8 mobile phase, are shown in FIG. 19. Further analyses were conducted in the same manner as in Example 10, and the pH-dependent dissociation rates for each clone are shown in Table 10.

[Table 10] Comparison of Dissociation Rate Constants of pH-Dependently-Binding Clones from Membrane-Type IL-6 Receptor, SR344

Sample	$k_d$ (1/s) pH7.4	$k_d$ (1/s) pH5.8	pH Dependency $k_d(\text{pH}5.8)/k_d(\text{pH}7.4)$
Fv4-IgG1	4.7E-04	2.6E-03	5.56
Fv4-IgG2	1.0E-03	1.8E-02	16.99
Fv4-M58	5.4E-04	9.5E-03	17.64
Fv4-M73	5.1E-04	5.1E-03	10.06

[0353] As a result of calculating the pH dependency for each clone, the pH dependencies of Fv4-IgG1, Fv4-IgG2, Fv4-M58, and Fv4-M73 to SR344 were 5.6-fold, 17.0-fold, 17.6-fold, and 10.1-fold, respectively; thus, Fv4-IgG2, Fv4-M58, and Fv4-M73 all demonstrated higher pH-dependent dissociation from the membrane-type IL-6 receptor than Fv4-IgG1.

[0354] Based on the results of analyzing binding to the soluble IL-6 receptor and binding to the membrane-type IL-6 receptor using the variable region of Fv4, it was found that substitution of the constant region from IgG1 to IgG2, M58, or M73 could improve the pH-dependent binding to the membrane-type IL-6 receptor, without causing a change in the affinity to the soluble IL-6 receptor. This was considered to similarly hold for Fv1, Fv2, and Fv3.

[Example 13] PK/PD Test of pH-Dependently-Binding Antibodies Having Optimized Constant Region Using Human IL-6 Receptor Transgenic Mice

[0355] The pharmacokinetics of Fv4-IgG1, Fv4-IgG2, and Fv4-M58 prepared in Example 13 were evaluated using the human IL-6 receptor transgenic mice (hIL-6R tg mice) used in Example 8 to examine the effects of the constant region on the pharmacokinetics. WT, Fv4-IgG1, Fv4-IgG2, or Fv4-M58 was administered to the hIL-6R tg mice by single-dose intravenous administration at 25 mg/kg, and then, measurement of the plasma concentrations of each antibody was

carried out in the same manner as in Example 8. The time courses of plasma concentrations of WT, Fv4-IgG1, Fv4-IgG2, and Fv4-M58 are shown in FIG. 20.

[0356] Similar to Example 11, the pharmacokinetics of Fv4-IgG1 was improved in comparison with WT, and the pharmacokinetics of Fv4-IgG2 and Fv4-M58 was further improved in comparison with Fv4-IgG1. Measurement as to the unbound IL-6 receptor concentrations, as measured in cynomolgus monkeys in Example 9, was carried out in the hIL-6R tg mice in this test using the same method. As a result, prolongation of the duration of neutralization of the soluble IL-6 receptor was confirmed for Fv4-IgG2 and Fv4-M58 in comparison to that of Fv4-IgG1 (data not shown). As indicated in Example 10, the pH-dependent binding to the membrane-type IL-6 receptor was improved for Fv4-IgG2 and Fv4-M58 as compared with Fv4-IgG1. Therefore, it was shown that improvement in the pH-dependent binding to the membrane-type IL-6 receptor and improvement in the pharmacokinetics and duration of neutralization of the soluble IL-6 receptor are possible by substituting the constant region from IgG1 to IgG2 or M58. On the basis of this finding, it was thought that the pharmacokinetics and duration of neutralization of the soluble IL-6 receptor, not only in the case of Fv4, but also in the cases of Fv1, Fv2, and Fv3, can be improved as compared to IgG1 by substituting the constant region from IgG1 to IgG2 or M58.

[Example 14] Construction of pH-Dependently-Binding Antibodies Having Optimized Variable and Constant Regions

[0357] VH2-M71 (SEQ ID NO: 36) and VH2-M73 (SEQ ID NO: 37), having M71 and M73 for the constant region of VH2-IgG1, and VH4-M71 (SEQ ID NO: 38) and VH4-M73 (SEQ ID NO: 39), having M71 and M73 for the constant region of VH4-IgG1, were constructed using the same method as described above.

[0358] Fv1-M71 using VH2-M71 for the H chain and VL2-CK for the L chain, Fv1-M73 using VH2-M73 for the H chain and VL2-CK for the L chain, Fv3-M71 using VH4-M71 for the H chain and VL1-CK for the L chain, and Fv3-M73 using VH4-M73 for the H chain and VL1-CK for the L chain, were expressed and purified. Expression and purification were carried out using the method described in Example 1.

#### Analyses of Binding of pH-Dependent-Binding Antibodies Having Optimized Variable and Constant Regions to Soluble IL-6 Receptor

[0359] The association to and dissociation from SR344 were observed on a real-time basis using the same method as Example 10 for the eleven types of antibodies, humanized PM1 antibody (wild type: WT) and H3pI/L73-IgG1, Fv1-M71, Fv1-M73, Fv2-IgG1, Fv3-M71, Fv3-M73, Fv4-IgG1, Fv4-IgG2, Fv4-M58, and Fv4-M73, constructed as described above. The association rate constants  $k_a$  (1/Ms) and dissociation rate constants  $k_d$  (1/s) were calculated by analysis in the same manner, and the dissociation constants  $K_D$  (M) were calculated on the basis of those values (Table 11).

[Table 11] Comparison of Dissociation Rate Constants of pH-Dependently-Binding Clones from Soluble IL-6 Receptor, SR344

Sample	$k_a$ (1/Ms)	$k_d$ (1/s)	$K_D$ (M)
WT	4. 0E+05	1. 1E-03	2. 7E-09
H3pI/L73	4. 1E+05	5. 9E-04	1. 4E-09
Fv1-M71	5. 5E+05	5. 4E-04	9. 7E-10
Fv1-M73	6. 1E+05	5. 5E-04	9. 1E-10
Fv2-IgG1	3. 9E+05	7. 7E-04	2. 0E-09
Fv3-M71	7. 8E+05	8. 2E-04	1. 1E-09
Fv3-M73	8. 5E+05	8. 7E-04	1. 0E-09
Fv4-IgG1	7. 2E+05	1. 0E-03	1. 4E-09
Fv4-IgG2	9. 6E+05	1. 2E-03	1. 3E-09
Fv4-M58	8. 3E+05	1. 1E-03	1. 4E-09
Fv4-M73	7. 5E+05	1. 0E-03	1. 4E-09

[0360] All of the resulting ten types of pH-dependent-binding clones were found to have dissociation constants (affinity,  $K_D$  values) to the soluble IL-6 receptor equal to or stronger than that of WT.

# Analyses of Binding of pH-Dependently-Binding Antibodies Having Optimized Variable and Constant Regions to Membrane-Type IL-6 Receptor

**[0361]** Antigen-antibody reactions to the membrane-type IL-6 receptor at pH 5.8 and pH 7.4 were observed in the same manner as in Example 10 using Biacore T100 (GE Healthcare) for the eleven types of antibodies, humanized PM1 antibody (wild type: WT) and H3pl/L73-IgG1, Fv1-M71, Fv1-M73, Fv2-IgG1, Fv3-M71, Fv3-M73, Fv4-IgG1, Fv4-IgG2, Fv4-M58, and Fv4-M73, prepared as described above. The pH-dependently-binding clones were injected under the condition of pH 7.4 to allow them to bind to SR344, and then, the pH-dependent dissociation of each clone at the pH of the mobile phase, pH 5.8, was observed. The results are shown in FIG. 21 (results for Fv1-M71, Fv1-M73, Fv3-M71, and Fv3-M73 are shown in FIG. 21, while results for other clones are shown in FIGs. 17 and 19). Analyses were carried out in the same manner as in Example 10 and the pH dependencies of the dissociation rate constants of all of the eleven types of clones are shown in Table 12.

[Table 12] pH Dependencies of Dissociation Rate Constants of pH-Dependently-Binding Clones from Membrane-Type IL-6 Receptor, SR344

Sample	pH7.4 kd (1/s)	pH5.8 kd (1/s)	pH Dependency kd(pH5.8)/kd(pH7.4)
WT	2.5E-04	2.5E-04	1.00
H3pl/L73	2.6E-04	6.7E-04	2.59
Fv1-M71	6.1E-04	6.9E-03	11.29
Fv1-M73	3.7E-04	3.2E-03	8.80
Fv2-IgG1	3.4E-04	2.4E-03	7.18
Fv3-M71	9.1E-04	9.7E-03	10.74
Fv3-M73	4.9E-04	5.3E-03	10.88
Fv4-IgG1	4.7E-04	2.6E-03	5.56
Fv4-IgG2	1.0E-03	1.8E-02	16.99
Fv4-M58	5.4E-04	9.5E-03	17.64
Fv4-M73	5.1E-04	5.1E-03	10.06

**[0362]** The ten types of obtained pH-dependently-binding clones demonstrated pH-dependent binding ability to the membrane-type IL-6 receptor. Moreover, all of Fv1-M71, Fv1-M73, Fv2-IgG1, Fv3-M71, Fv3-M73, Fv4-IgG1, Fv4-IgG2, Fv4-M58, and Fv4-M73 were found to demonstrate improved pH-dependent binding to the membrane-type IL-6 receptor, in comparison with H3pl/L73-IgG1, for which the time until the antibody disappears from the plasma as well as the time where the soluble IL-6 receptor and membrane-type IL-6 receptor are bound by the antibody in the body were found to be prolonged considerably in comparison with WT, as shown in cynomolgus monkeys in Example 9.

## [Example 15] PK/PD Test of pH-Dependently-Binding Antibodies Having Optimized Variable and Constant Regions Using Cynomolgus Monkeys

### Construction of Known High-Affinity Anti-IL-6 Receptor Antibody

**[0363]** A mammalian cell expression vector was constructed to express the high-affinity anti-IL-6 receptor antibody VQ8F11-21 hIgG1 described in US 2007/0280945 A1 (US 2007/0280945 A1, amino acid sequences 19 and 27), as a known high-affinity anti-IL-6 receptor antibody. An antibody variable region was constructed by PCR combining synthetic oligo-DNAs (assembly PCR). The constant region was amplified by PCR from the expression vector used in Example 1. The antibody variable region and antibody constant region were ligated by assembly PCR and inserted into a vector for expression in mammals. The resulting H chain and L chain DNA fragments were inserted into mammalian cell expression vectors to construct the H chain expression vector and L chain expression vector of interest. The nucleotide sequences of the resulting expression vectors were determined by a method known among persons with ordinary skill in the art. Expression and purification were carried out using the constructed expression vectors. Expression and purification were carried out using the method described in Example 1 to obtain the high-affinity anti-IL-6 receptor antibody (high affinity Ab).



PK/PD Test in Cynomolgus Monkeys

**[0364]** The pharmacokinetics and pharmacological efficacy were evaluated in cynomolgus monkeys for the pH-dependently-binding antibodies H3pl/L73-IgG1 and Fv1-M71, Fv1-M73, Fv2-IgG1, Fv3-M73, and Fv4-M73 and the known high-affinity anti-IL-6 receptor antibody (high affinity Ab). H3pl/L73-IgG1, Fv1-M71, Fv1-M73, Fv2-IgG1, Fv3-M73, or Fv4-M73 was administered to Cynomolgus monkeys by single-dose intravenous administration at 0.5 mg/kg, while the high affinity Ab was administered by single-dose intravenous administration at 1.0 mg/kg. Blood samples were collected before administration and over time. The plasma concentration of each antibody was measured in the same manner as in Example 9. The time courses of plasma concentrations of H3pl/L73-IgG1, Fv1-M71, Fv1-M73, Fv2-IgG1, Fv3-M73, and Fv4-M73, as well as the high affinity Ab are shown in FIG. 21. In order to evaluate the pharmacological efficacy in terms of the degree to which the cynomolgus monkey membrane-type IL-6 receptor is neutralized, cynomolgus monkey IL-6 was administered subcutaneously into the lower backs of the animals daily at 5 µg/kg from day 3 to day 10 (from day 6 to day 10 with respect to the high affinity Ab) after the administration of antibody, in the same manner as in Example 9. The CRP concentration of each animal was measured 24 hours after each administration. The time courses of CRP concentrations with the administration of each antibody are shown in FIG. 22. In order to evaluate the pharmacological efficacy in terms of the degree of neutralization of cynomolgus monkey soluble IL-6 receptor, the concentration of the unbound cynomolgus monkey soluble IL-6 receptor in cynomolgus monkey plasma was measured in the same manner as Example 9. The time courses of the unbound cynomolgus monkey soluble IL-6 receptor concentrations with the administration of each antibody are shown in FIG. 23.

**[0365]** As a result, the antibody concentrations in plasma were maintained high for each of Fv1-M71, Fv1-M73, Fv2-IgG1, Fv3-M73 and Fv4-M73 in comparison with H3pl/L73-IgG1, while the concentrations of CRP and the unbound cynomolgus monkey soluble IL-6 receptor were maintained at low levels. Namely, this result showed that the time where the membrane-type and soluble IL-6 receptors are bound by the antibody (or in other words, the duration of neutralization) was prolonged by the antibodies in comparison with H3pl/L73-IgG1.

**[0366]** In addition, these pH-dependently-binding anti-IL-6 receptor antibodies were confirmed for their neutralization effects and sustained efficacy equal to or greater than that of the known high-affinity anti-IL-6 receptor antibody (high affinity Ab) administered at 1.0 mg/kg, at only half the dosage thereof, i.e., at 0.5 mg/kg. Therefore, the pH-dependently-binding antibodies were elucidated to have the neutralization effects and sustained efficacy superior to those of the known high-affinity anti-IL-6 receptor antibody.

**[0367]** Those antibodies shown in Table 12 for which PK/PD tests were not carried out using cynomolgus monkeys as in this test, have also been confirmed to demonstrate improved pH-dependent binding to the membrane-type IL-6 receptor in comparison with H3pl/L73-IgG1. Therefore, the time during which the membrane-type and soluble IL-6 receptor are bound by the antibodies (or in other words, the duration of neutralization and sustained neutralization effects) are also thought to be prolonged for the antibodies in comparison with H3pl/L73-IgG1.

**[0368]** In Example 9, for H3pl/L73-IgG1, the time until the antibody disappears from the plasma as well as the time where the soluble IL-6 receptor and membrane-type IL-6 receptor are bound by the antibody in the body (sustained neutralization effects) were found to be prolonged considerably as compared with WT. Fv1-M71, Fv1-M73, Fv2-IgG1, Fv3-M71, Fv3-M73, Fv4-IgG1, Fv4-IgG2, Fv4-M58, and Fv4-M73, having superior sustained neutralization effects to H3pl/L73-IgG1, are therefore thought to have remarkably improved sustained neutralization effects as compared with WT.

**[0369]** In contrast to anti-IL-6 receptor antibodies, the pH-dependent-binding anti-IL-6 receptor antibodies that are made to strongly bind to the antigen at the pH in the plasma of pH 7.4 but only weakly bind to the antigen at the pH in endosomes of pH 5.8, make it possible to reduce the patient dosage and administration frequency of the anti-IL-6 receptor antibody, and as a result, they can considerably reduce the total administration amount. Therefore, the pH-dependently-binding anti-IL-6 receptor antibodies are thought to be extremely superior as a pharmaceutical for use as an IL-6 antagonist.

[Example 16] Construction of pH-Dependently Binding Anti-IL-6 AntibodyExpression and Purification of Anti-IL-6 Antibody

**[0370]** In Examples 1 to 15, a plurality of humanized anti-IL-6 receptor antibodies that pH-dependently bind to the IL-6 receptor were successfully created by imparting the dependency through introducing histidine substitutions and the like into the variable region, in particular, the CDR sequences of the humanized anti-IL-6 receptor antibodies. It was found that all of these antibodies repeatedly bind to the IL-6 receptor and demonstrate a considerable improvement in PK/PD.

**[0371]** Therefore, it was confirmed that the pH-dependent ability of an antibody to bind to an antigen could be conferred to another antibody that binds to an antigen other than the IL-6 receptor using a similar method. Human IL-6 was selected as the antigen, and an anti-IL-6 antibody including the H chain (WT) (amino acid sequence: SEQ ID NO: 62) and L chain

(WT) (amino acid sequence: SEQ ID NO: 63), which binds to IL-6 as described in WO 2004/039826, ("Anti-IL6 wild type") was constructed. Using a method known to those skilled in the art gene fragments encoding the antibody amino acid sequences of interest were inserted into mammalian cell expression vectors to construct the H chain expression vector and L chain expression vector of interest. The nucleotide sequences of the resulting expression vectors were determined using a method known to a skilled person. Anti-IL6 wild type was expressed and purified by the method described in Example 1.

#### Construction of pH-Dependent Anti-IL-6 Antibody

**[0372]** To confer the pH-dependent ability of the antibody to bind to IL-6, histidine substitutions were introduced into the amino acids in CDR of the anti-IL-6 antibody (Anti-IL6 wild type) including the H chain (WT) (amino acid sequence: SEQ ID NO: 62) and L chain (WT) (amino acid sequence: SEQ ID NO: 63). By substituting histidine in the CDR amino acids and subsequently screening, several clones that demonstrate the pH-dependent binding were obtained. The binding at pH 5.5 was significantly reduced as compared with the binding at pH 7.4. The positions of histidine substitution in the pH-dependent clones are shown in Table 13. The examples include "Anti-IL6 clone 1" including the H chain (c1) (amino acid sequence: SEQ ID NO: 64) and L chain (c1) (amino acid sequence: SEQ ID NO: 65), and "Anti-IL-6 clone 2" including the H chain (c1) (amino acid sequence: SEQ ID NO: 64) and L chain (c2) (amino acid sequence: SEQ ID NO: 66). Anti-IL6 clone 1 and Anti-IL-6 clone 2 were expressed and purified by the method described in Example 1.

[Table 13] Positions of Histidine Substitution in pH-dependent clones

H32, H59, H61, H99

L53, L54, L90, L94

#### Analysis of Binding of pH-Dependent Clones to Human IL-6

**[0373]** Kinetic analysis of antigen-antibody reactions at pH 5.5 and pH 7.4 was carried out using Biacore T100 (GE Healthcare) for the three types of antibodies prepared as mentioned above: Anti-IL6 wild type, Anti-IL6 clone 1, and Anti-IL-6 clone 2 (buffer: DPBS(-) (pH 7.4 or pH 5.5), 150 mM NaCl). The antibodies were bound to a sensor chip on which recomb-protein A/G (Pierce) was immobilized by amine coupling, and then human IL-6 (Toray) adjusted to an appropriate concentration was injected on the chip as an analyte. All the measurements were carried out at 37°C. Association rate constants  $k_a$  (1/Ms) and dissociation rate constants  $k_d$  (1/s) were calculated using Biacore T100 Evaluation Software (GE Healthcare), and dissociation constants  $K_D$  (M) were calculated based on those values (Table 14). Furthermore, the ratio of affinity at pH 5.5 and pH 7.4 was calculated for each clone to evaluate the pH-dependent binding.

[Table 14] Comparison of Binding of pH-Dependent Clones to IL-6

Sample	pH	$k_a$ (1/Ms)	$k_d$ (1/s)	$K_D$ (M)	$K_D$ (pH5.5)/ $K_D$ (pH7.4)
Wild type	pH7.4	2.05E+07	3.91E-04	1.91E-11	0.8
	pH5.5	1.52E+07	2.45E-04	1.61E-11	
clone1	pH7.4	1.07E+07	4.71 E-03	4.38E-10	10.3
	pH5.5	2.05E+06	9.26E-03	4.52E-09	
clone2	pH7.4	8.96E+06	2.63E-03	2.94E-10	13.5
	pH5.5	2.76E+06	1.10E-02	3.98E-09	

**[0374]** The ratio of affinity at pH 5.5 and pH 7.4 ( $(K_D)(pH\ 5.5)/(K_D)(pH\ 7.4)$ ) calculated, which indicates the pH-dependent binding to human IL-6, was 0.8, 10.3, and 13.5 for Anti-IL6 wild type, Anti-IL6 clone 1, and Anti-IL6 clone 2, respectively. That is, the pH-dependent binding ability of each clone is more than 10 times greater than that of WT. The sensorgrams of Anti-IL-6 clone 2 at pH 7.4 and pH 5.5 are shown in FIG. 26.

**[0375]** Thus, it was shown that, as in the case of the anti-IL-6 receptor antibodies, pH-dependently binding anti-IL-6 antibodies that bind to the antigen strongly under plasma neutral conditions, but weakly under intraendosomal acidic conditions can be constructed by introducing histidine substitutions and the like mainly into the CDR amino acid sequences. As indicated in Examples 1 to 15, an anti-IL-6 receptor antibody that has the pH-dependent binding ability repeatedly binds to the IL-6 receptor and PK/PD is remarkably improved. That is, it was suggested that Anti-IL-6 clone 1 and Anti-IL-6 clone 2, which have the pH-dependent binding ability, repeatedly bind to more antigens with significantly

improved PK/PD, as compared with Anti-IL6 wild type.

#### [Example 17] Construction of pH-Dependently Binding Anti-IL-31 Receptor Antibody

##### Expression and Purification of Anti-IL-31 Receptor Antibody

[0376] In Examples 1 to 15, a plurality of humanized anti-IL-6 receptor antibodies that pH-dependently bind to the IL-6 receptor were successfully created by conferring the pH dependency through introducing histidine substitutions and the like into the variable region, in particular, the CDR sequences of the humanized anti-IL-6 receptor antibodies. It was found that all of these antibodies repeatedly bind to the IL-6 receptor and demonstrate a considerable improvement of PK/PD.

[0377] Therefore, it was confirmed that the pH dependent ability of an antibody to bind to an antigen could be conferred to another antibody that binds to an antigen other than the IL-6 receptor using a similar method. Mouse IL-31 receptor was selected as the antigen, and an anti-IL-31 receptor antibody including the H chain (WT) (amino acid sequence: SEQ ID NO: 67) and L chain (WT) (amino acid sequence: SEQ ID NO: 68), which binds to the mouse IL-31 receptor as described in WO 2007/142325, ("Anti-IL31R wild type") was constructed. Using a method known to those skilled in the art, gene fragments encoding the amino acid sequences of interest were inserted into mammalian cell expression vectors to construct the H chain expression vector and L chain expression vector of interest. The nucleotide sequences of the resulting expression vectors were determined using a method known to a skilled person. Anti-IL31R wild type was expressed and purified by the method described in Example 1.

##### Construction of pH-Dependent Anti-IL-31 Receptor Antibody

[0378] To confer the pH-dependent ability of the antibody to bind to the IL-31 receptor, histidine substitutions were introduced into the amino acids of CDR of the anti-IL-31 receptor antibody (Anti-IL31R wild type) including the H chain (WT) (amino acid sequence: SEQ ID NO: 67) and L chain (WT) (amino acid sequence: SEQ ID NO: 68). By histidine substitutions in the CDR amino acids and subsequent screening, several clones that demonstrate the pH-dependent binding were obtained. The binding at pH 5.5 was significantly reduced as compared with the binding at pH 7.4. The position of histidine substitution in the pH-dependent clones is shown in Table 15. An example is "Anti-IL31R clone 1" including the H chain (c1) (amino acid sequence: SEQ ID NO: 69) and L chain (WT). Anti-IL31R clone 1 was expressed and purified using the method described in Example 1.

[Table 15] Position of Histidine Substitution in pH-dependent clones  
H33

##### Analysis of Binding of pH-Dependent Clones to Soluble IL-31 Receptor

[0379] Kinetic analysis of antigen-antibody reactions at pH 5.5 and pH 7.4 was carried out using Biacore T100 (GE Healthcare) for the two types of antibodies prepared as mentioned above: Anti-IL31R wild type and Anti-IL31R clone 1 (buffer: DPBS(-) (pH 7.4 or pH 5.5), 150 mM NaCl, 0.01% Tween 20, 0.02% NaN<sub>3</sub>). The antibodies were bound to a sensor chip on which recomb-protein A/G (Pierce) was immobilized by amine coupling, and then the soluble mouse IL-31 receptor (prepared according to the method described in WO 2007/142325) adjusted to an appropriate concentration was injected therein as an analyte. All the measurements were carried out at 25°C. Association rate constants  $k_a$  (1/Ms) and dissociation rate constants  $k_d$  (1/s) were calculated using Biacore T100 Evaluation Software (GE Healthcare), and dissociation constants  $K_D$  (M) were calculated based on those values (Table 16). Furthermore, the ratio of affinity at pH 5.5 and pH 7.4 was calculated for each clone to evaluate the pH-dependent binding.

[Table 16] Comparison of Binding of pH-Dependent Clones to Mouse IL-31 Receptor

Sample	pH	$k_a$ (1/Ms)	$k_d$ (1/s)	$K_D$ (M)	$K_D$ (pH5.5)/ $K_D$ (pH7.4)
Wild type	pH7.4	1.40E+05	3.40E-03	2.30E-08	3.2
	pH5.5	5.10E+05	3.80E-03	7.40E-08	
clone1	pH7.4	1.70E+05	3.30E-03	2.20E-08	1000.0
	pH5.5	1.10E+03	2.40E-02	2.20E-05	

[0380] The ratio of affinity at pH 5.5 and pH 7.4 ( $(KD)(pH\ 5.5)/(KD)(pH\ 7.4)$ ) calculated, which indicates the pH-dependent binding to the mouse IL-31 receptor, was 3.2 and 1000 for Anti-IL31R wild type and Anti-IL31R clone 1, respectively. That is, the pH-dependent binding ability of Anti-IL31R clone 1 is about 300 times greater than that of WT. The sensorgrams for Anti-IL31R clone 1 at pH 7.4 and pH 5.5 are shown in FIG. 27.

[0381] Thus, it was shown that, as in the cases of the anti-IL-6 receptor antibodies and anti-IL-6 antibodies, pH-dependent binding anti-IL-31 receptor antibodies that bind to the antigen strongly under plasma neutral conditions, but weakly under intraendosomal acidic conditions can be constructed by introducing histidine substitutions and the like mainly into the CDR amino acid sequences. As indicated in Examples 1 to 15, an anti-IL-6 receptor antibody that has the pH-dependent binding ability repeatedly binds to the IL-6 receptor and PK/PD is remarkably improved. That is, it was suggested that Anti-IL31R clone 1, which has the pH-dependent binding ability, repeatedly binds to more antigens with significantly improved PK/PD, as compared with Anti-IL31R wild type.

#### [Example 18] Repetitive Binding to Antigen by pH-Dependently Binding Antibody

#### Expression and Purification of Antibody Administered to Mice

[0382] The four types of humanized IL-6 receptor antibodies described below were prepared. As the antibodies that do not pH-dependently bind to the IL-6 receptor, WT-IgG1 including H (WT) (amino acid sequence: SEQ ID NO: 9) and L (WT) (amino acid sequence: SEQ ID NO: 10), and H54/L28-IgG1 including H54 (amino acid sequence: SEQ ID NO: 70) and L28 (amino acid sequence: SEQ ID NO: 12) were expressed and purified using the method indicated in Example 1. As the antibodies that pH-dependently bind to the IL-6 receptor, H170/L82-IgG1 of Example 3 including H170 (amino acid sequence: SEQ ID NO: 4) and L82 (amino acid sequence: SEQ ID NO: 7), and Fv4-IgG1 of Example 10 including VH3-IgG1 (SEQ ID NO: 23) and VL3-CK (SEQ ID NO: 27), were expressed and purified using the method indicated in Example 1.

#### Analysis of Binding of Each Type of Antibody to Soluble IL-6 Receptor

[0383] Kinetic analysis of antigen-antibody reactions at pH 7.4 and pH 5.8 was carried out using Biacore T100 (GE Healthcare) for the four types of antibodies prepared: WT-IgG1, H54/L28-IgG1, H170/L82-IgG1, and Fv4-IgG1 (buffer: 10 mM MES (pH 7.4 or pH 5.8), 150 mM NaCl, 0.05% Surfactant-P20). The antibodies were bound to a sensor chip on which recomb-protein A/G (Pierce) was immobilized by amine coupling, and SR344 adjusted to an appropriate concentration was injected therein as an analyte. The association with and dissociation from SR344 of each type of antibody were observed on a real-time basis. All the measurements were carried out at 37°C. Association rate constants  $k_a$  (1/Ms) and dissociation rate constants  $k_d$  (1/s) were calculated using Biacore T100 Evaluation Software (GE Healthcare), and dissociation constants  $KD$  (M) were calculated based on those values (Table 17).

[Table 17] Comparison of Association Rates ( $k_a$ ), Dissociation Rates ( $k_d$ ), and Dissociation Constants of Each Type of Antibody Against Soluble IL-6 Receptor (SR344)

Sample	$k_a$ (1/Ms)	pH7.4		$k_a$ (1/Ms)	pH5.8		$k_d$ (pH5.8/ $k_d$ (pH7.4)	pH Dependency	
		$k_d$ (1/s)	$KD$ (M)		$k_d$ (1/s)	$KD$ (M)		$KD$ (pH5.8) / $KD$ (pH7.4)	
WT-IgG1	4.9E+05	9.4E-04	1.9E-09	8.9E+05	2.7E-03	3.1E-09	2.9	1.6	
H54/L28-IgG1	8.3E+05	1.4E-03	1.7E-09	2.4E+06	2.7E-03	1.1E-09	2.0	0.7	
H170/LB2-IgG1	6.7E+05	1.1E-03	1.6E-09	1.2E+05	1.3E-02	1.0E-07	11.4	61.9	
Fv4-IgG1	9.8E+05	9.5E-04	9.7E-10	1.4E+06	3.7E-02	2.6E-08	38.8	27.3	

[0384] The ratio of affinity ( $KD$ ) at pH 5.8 and pH 7.4 for each antibody was calculated. The  $KD$  ratio, which indicates the pH-dependent binding to SR344, was 1.6, 0.7, 61.9, and 27.3 for WT-IgG1, H54/L28-IgG1, H170/L82-IgG1, and Fv4-IgG1, respectively. In addition, the ratio of dissociation rate ( $k_d$ ) at pH 5.8 and pH 7.4 for each antibody was calculated. The  $k_d$  ratio, which indicates the pH-dependent dissociation rate for SR344, was 2.9, 2.0, 11.4, and 38.8 for WT-IgG1, H54/L28-IgG1, H170/L82-IgG1, and Fv4-IgG1, respectively. Thus, it was confirmed that H170/L82-IgG1 and Fv4-IgG1 demonstrate the pH-dependent binding, while the conventional antibodies WT-IgG1 and H54/L28-IgG1 hardly exhibit

the ability. In addition, since the affinity (KD) of these antibodies at pH 7.4 was nearly equal, their ability to bind to SR344 in the plasma was thought to be equivalent.

#### In Vivo Pharmacokinetics Test Using Mice

**[0385]** The pharmacokinetics of SR344 and the anti-human IL-6 receptor antibody were evaluated following administration of SR344 (human IL-6 receptor, prepared in Example 1) only or simultaneous administration of SR344 and the anti-human IL-6 receptor antibody to mice that do not express the human IL-6 receptor (C57BL/6J; the anti-human IL-6 receptor antibodies do not bind to the mouse IL-6 receptor). An SR344 solution (5 µg/mL) or a mixed solution containing SR344 and the anti-human IL-6 receptor antibody (5 µg/mL and 0.1 mg/mL, respectively) was administered into a caudal vein by single-dose administration at 10 mL/kg. Since the anti-human IL-6 receptor antibody was present in an adequate excess amount relative to SR344, it was thought that nearly all of the SR344 molecules were bound by the antibody. Blood samples were collected at 15 minutes, 2 hours, 8 hours, 1 day, 2 days, 3 days, 4 days, 7 days, 14 days, 21 days, and 28 days after administration. The collected blood samples were immediately centrifuged for 15 minutes at 15,000 rpm and 4°C to obtain the plasma. The plasma separated was stored in a freezer set to -20°C or lower until the time of measurement. Above-described WT-IgG1, H54/L28-IgG1, H170/L82-IgG1, and Fv4-IgG1 were used as the anti-human IL-6 receptor antibodies.

#### Measurement of Anti-Human IL-6 Receptor Antibody Plasma Concentration by ELISA

**[0386]** The concentration of human IL-6 receptor antibody in mouse plasma was measured by ELISA. Anti-human IgG (γ-chain specific) F(ab')<sub>2</sub> antibody fragment (Sigma) was dispensed onto a Nunc-ImmunoPlate MaxiSorp (Nalge Nunc International) and allowed to stand overnight at 4°C to prepare anti-human IgG-immobilized plates. Calibration curve samples having plasma concentrations of 0.8, 0.4, 0.2, 0.1, 0.05, 0.025, and 0.0125 µg/mL, and mouse plasma samples diluted 100-fold or more were prepared. 200 µL of 20 ng/mL SR344 was added to 100 µL of the calibration curve samples and plasma samples, and then the samples were allowed to stand for 1 hour at room temperature. Subsequently, the samples were dispensed into the anti-human IgG-immobilized plates, and allowed to stand for 1 hour at room temperature. Then, Biotinylated Anti-Human IL-6R Antibody (R&D) was added to react for 1 hour at room temperature. Subsequently, Streptavidin-PolyHRP80 (Stereospecific Detection Technologies) was added to react for 1 hour at room temperature, and chromogenic reaction was carried out using TMP One Component HRP Microwell Substrate (BioFX Laboratories) as a substrate. After stopping the reaction with 1 N sulfuric acid (Showa Chemical), the absorbance at 450 nm was measured by a microplate reader. The concentration in mouse plasma was calculated from the absorbance of the calibration curve using the analytical software SOFTmax PRO (Molecular Devices). The time course of plasma concentration after intravenous administration as measured by this method is shown in FIG. 28.

#### Measurement of SR344 Plasma Concentration by Electrochemiluminescence

**[0387]** The concentration of SR344 in mouse plasma was measured by electrochemiluminescence. SR344 calibration curve samples adjusted to concentrations of 2000, 1000, 500, 250, 125, 62.5, and 31.25 µg/mL, and mouse plasma samples diluted 50-fold or more were prepared. The samples were mixed with a solution of Monoclonal Anti-human IL-6R Antibody (R&D) ruthenium-labeled with Sulfo-Tag NHS Ester (Meso Scale Discovery), Biotinylated Anti-human IL-6R Antibody (R&D), and WT-IgG1, and then allowed to react overnight at 37°C. The final concentration of WT-IgG1 was 333 µg/mL, which is in excess of the concentration of anti-human IL-6 receptor antibody contained in the samples, for the purpose of binding nearly all of the SR344 molecules in the samples to WT-IgG1. Subsequently, the samples were dispensed into an MA400 PR Streptavidin Plate (Meso Scale Discovery), and allowed to react for 1 hour at room temperature, and washing was performed. Immediately after Read Buffer T (x4) (Meso Scale Discovery) was dispensed, the measurement was performed by the Sector PR 400 Reader (Meso Scale Discovery). The SR344 concentration was calculated based on the response of the calibration curve using the analytical software SOFTmax PRO (Molecular Devices). The time course of SR344 plasma concentration after intravenous administration as measured by this method is shown in FIG. 29.

#### Effects of pH-Dependent Binding

**[0388]** With respect to the time course of antibody concentration of WT-IgG1 and H54/L28-IgG1, which do not demonstrate the pH-dependent binding, and H170/L82-IgG1 and Fv4-IgG1, which demonstrate the pH-dependent binding, the time course of concentration was roughly identical for WT-IgG1, H54/L28-IgG1 and Fv4-IgG1, while H170/L82-IgG1 was eliminated slightly more rapidly. The data of the time course of plasma concentration was analyzed by the pharmacokinetics analysis software WinNonlin (Pharsight). The half-lives in the plasma of WT-IgG1, H54/L28-IgG1, Fv4-IgG1,



and H170/1.28-IgG1 were 21.0, 28.8, 26.2, and 7.5 days, respectively.

**[0389]** As described in Example 2, when the antigen is a soluble antigen, an administered antibody binds to the antigen in the plasma, and is retained in the plasma in the form of an antigen-antibody complex. Generally, in contrast to extremely long plasma retention time of an antibody (the elimination rate is extremely low) due to the function of FcRn, the plasma retention time of an antigen is short (the elimination rate is high). Thus, an antigen that is bound to an antibody has a prolonged plasma retention time similar to that of an antibody (the elimination rate is extremely low). Similarly, when the antigen of humanized IL-6 receptor antibody, SR344 (soluble human IL-6 receptor), was administered alone, SR344 was extremely rapidly eliminated (plasma half-life: 0.2 days). However, in the case of concurrent administration of SR344 with a conventional antibody, WT-IgG1 or H54/L28-IgG1, which does not demonstrate the pH-dependent binding, the elimination rate of SR344 was considerably reduced, and the plasma retention time of SR344 was prolonged (plasma half-life: 5.3 days for WT-IgG1, 6.3 days for H54/L28-IgG1). This is because nearly all of the SR344 molecules were bound by the antibodies administered together, and thus SR344 bound by the antibodies had a prolonged plasma retention time similar to that of the antibody, due to the function of FcRn as described above.

**[0390]** In the case of concurrent administration of SR344 with the H170/L82-IgG1 or Fv4-IgG1 antibody, which demonstrates the pH-dependent binding, the elimination of SR344 was significantly rapid (plasma half-life: 1.3 days for H170/L82-IgG1, 0.6 days for Fv4-IgG1), as compared to the case of concurrent administration with WT-IgG1 or H54/L28-IgG1. This tendency was particularly prominent for Fv4-IgG1. Since the affinity of Fv4-IgG1 at pH 7.4 is equivalent to or stronger than that of WT-IgG1 and H54/L28-IgG1, it is thought that nearly all of the SR344 molecules were bound to Fv4-IgG1. Even though Fv4-IgG1 demonstrates equivalent or slightly longer plasma retention and slower elimination compared to WT-IgG1 and H54/L28-IgG1, the elimination of SR344 bound to Fv4-IgG1 was extremely rapid. This can be explained by the concept of the present technology shown in FIG. 4. In the case of conventional antibodies that do not demonstrate the pH-dependent binding, an antibody-soluble antigen complex is taken up into endosomes by pinocytosis in the plasma, and binds to FcRn expressed in endosomes under intraendosomal acidic conditions. Since the antibody-soluble antigen complex bound to FcRn transfers to the cell surface as it is, and again returns to the plasma, the antigen bound to the antibody has a prolonged plasma retention time similar to that of the antibody (the elimination is extremely slow). On the other hand, in the case of antibodies that demonstrate the pH-dependent binding, the antigen dissociates from the antibody under intraendosomal acidic conditions, and thus only the antibody binds to FcRn and returns again to the plasma. Since the antigen dissociated from the antibody is degraded in lysosomes without returning to the plasma, the elimination of antigen is extremely rapid as compared to the case of antibodies that do not demonstrate the pH-dependent binding. Namely, in the case of concurrent administration of SR344 with the WT-IgG1 or H54/L28-IgG1 antibody, which does not demonstrate the pH-dependent binding, the elimination of SR344 is slow to the similar degree as the antibody, since SR344 binds to WT-IgG1 or H54/L28-IgG1 both in plasma and endosomes. In contrast, in the case of concurrent administration of SR344 with the H170/L82-IgG1 or Fv4-IgG1 antibody, which demonstrates the pH-dependent binding, the elimination of SR344 is extremely rapid, since SR344 dissociates from the antibody in the intraendosomal low-pH environment. That is, since the H170/L28-IgG1 and Fv4-IgG1 antibodies, which demonstrate the pH-dependent binding, dissociate from SR344 in the intraendosomal low-pH environment, the majority of H170/L82-IgG1 or Fv4-IgG1 that has returned again to the plasma with FcRn is thought to be not bound to SR344. Thus, as shown in FIG. 4, it was revealed that, by dissociating from an antigen in the intraendosomal low-pH environment and returning to the plasma with FcRn without binding to the antigen, an antibody that demonstrates the pH-dependent binding can again bind to an antigen in the plasma. It was also shown that, by repeating this process, the antibody that demonstrates the pH-dependent binding can repeatedly bind to multiple antigens. This is consistent with the Biacore data shown in Example 7, demonstrating that pH-dependent antibodies can repeatedly bind to antigens. Thus, by enhancing the pH-dependent binding of an antibody to an antigen, the number of times of repetitive antigen binding can be increased.

**[0391]** When the antigen is a soluble antigen, and the antigen binds to an antibody under plasma neutral conditions, but dissociates from the antibody in endosomes and the antibody returns to the plasma with FcRn, the antibody can again bind to an antigen under plasma neutral conditions. Thus, an antibody that has the ability to dissociate from an antigen under intraendosomal acidic conditions can bind to antigens multiple times. As compared to when an antigen bound to an antibody does not dissociate from the antibody in endosomes (*i.e.*, the antigen bound to the antibody returns to the plasma), if an antigen bound to an antibody dissociates from the antibody in endosomes, the plasma elimination rate of the antigen is increased, since the antigen is transported to lysosomes and degraded. Thus, the plasma elimination rate of an antigen can be used as an index to determine whether an antibody can bind to the antigen multiple times. Determination of the plasma elimination rate of an antigen can be performed, for example, by administering an antigen and an antibody *in vivo*, and then measuring the plasma antigen concentration after the administration, as shown in the Examples.

**[0392]** An antibody that demonstrates the pH-dependent binding can repeatedly bind to multiple antigens in contrast to the case of a conventional antibody that does not demonstrate the pH-dependent binding. Thus, the amount of antibody administered can be considerably reduced, and the administration intervals can be greatly prolonged.

**[0393]** The repetitive binding to multiple antigens of this mechanism is based on pH-dependent antigen-antibody

reaction. Thus, regardless of the type of antigen, if an antibody demonstrating the pH-dependent binding that binds to an antigen at pH 7.4 of plasma but dissociates from the antigen at intraendosomal acidic pH can be constructed, then such an antibody can repeatedly bind to multiple antigens. Accordingly, the present technology is useful in that it can be applied not only to antibodies to the IL-6 receptor, IL-6, and the IL-31 receptor, but generally to any antibody to any antigen, regardless of the type of antigen.

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## SEQUENCE LISTING

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50	Asp Ser Val Thr Ile Thr Cys Gln Ala Ser Gln Asp Ile Ser Ser His		
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5 Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Glu Leu Leu Ile  
35 40 45

10 Tyr Tyr Gly Ser His Leu His His Gly Val Pro Ser Arg Phe Ser Gly  
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15 Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Glu Ala  
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20 Glu Asp Ala Ala Thr Tyr Tyr Cys Gly Gln Gly His Arg Leu Pro Tyr  
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55 Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Tyr Ser Ile Thr Ser Asp

EP 2 275 443 A1

	20	25	30
5	His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Arg Gly Leu Glu Trp		
	35	40	45
10	Ile Gly Tyr Ile Ser Tyr Ser Gly Ile Thr Thr Tyr Asn Pro Ser Leu		
	50	55	60
15	Lys Ser Arg Val Thr Met Leu Arg Asp Thr Ser Lys Asn Gln Phe Ser		
20	65	70	75 80
25	Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr Cys		
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30	Ala Arg Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr Trp Gly Gln Gly		
	100	105	110
35	Ser Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe		
	115	120	125
40	Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu		
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50	Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp		
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55	Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu		

## EP 2 275 443 A1

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EP 2 275 443 A1

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Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys  
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Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr  
340 345 350

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Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr  
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Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu  
370 375 380

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Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu  
385 390 395 400

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Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys  
405 410 415

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Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu  
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Tyr Tyr Thr Ser Arg Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly  
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Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Gln Pro  
65 70 75 80

Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Gly Asn Thr Leu Pro Tyr  
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Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala  
100 105 110

5 Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly  
115 120 125

10 Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala  
130 135 140

15 Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln  
145 150 155 160

20 Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser  
165 170 175

25 Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr  
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35 Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser  
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Thr Leu Ser Leu Thr Cys Ala Val Ser Gly Tyr Ser Ile Ser Asp Asp  
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His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp  
 35 40 45

Ile Gly Tyr Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Ser Leu  
 50 55 60

Lys Gly Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser  
 65 70 75 80

Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Ala Tyr Tyr Cys  
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Ala Arg Val Leu Ala Arg Ile Thr Ala Met Asp Tyr Trp Gly Glu Gly  
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Thr Leu Val Thr Val Ser Ser  
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Asp Ser Val Thr Ile Thr Cys Gln Ala Ser Gln Asp Ile Ser Ser Tyr  
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Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Glu Leu Leu Ile  
35 40 45

Tyr Tyr Gly Ser Glu Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly  
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Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Glu Ala  
65 70 75 80

Glu Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Gly Asn Ser Leu Pro Tyr  
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Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Glu  
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 25 cctggagaag gtcttgagtg gattggatac attagttata gtggaatcac aaactataat 240  
 30 ccatctctca aaggcagagt gacaatatcg agagacacca gcaagaacca gttcagcctg 300  
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 50 tgcaacgtga atcacaagcc cagcaacacc aagggtggaca agaaagtiga gcccaaattc 720  
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10 taccgtgtgg tcagcgtcct caccgtcctg caccaggact ggctgaatgg caaggagtag 1020

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gagtgggaga gcaatgggca gccggagaa aactacaaga ccacgcctcc cgtgctggac 1260

25 tccgacggct ctttcttct ctacagcaag ctaccgtgg acaagagcag gtggcagcag 1320

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 5 agattcagcg gtagcggtag cggtaacgac ttcaccttca coacagcag cctcgaggca 240  
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45           His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp  
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10	Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Ala Tyr Tyr Cys			
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15	Ala Arg Phe Leu Ala Arg Ile Thr Ala His Asp His Trp Gly Glu Gly			
		100	105	110
20	Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe			
		115	120	125
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30	Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu			
		130	135	140
35	Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp			
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40	Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu			
		165	170	175
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50	Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser			
		180	185	190
55	Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro			
		195	200	205

EP 2 275 443 A1

5	Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys	210	215	220
10	Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro	225	230	235 240
15	Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser	245	250	255
20	Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp	260	265	270
25	Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn	275	280	285
30	Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val	290	295	300
35	Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu	305	310	315 320
40	Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys	325	330	335
45	Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr	340	345	350
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55				



5 Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr  
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10 Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu  
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15 Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu  
385 390 395 400

20 Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys  
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25 Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu  
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35 Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly  
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Thr Leu Ser Leu Thr Cys Ala Val Ser Gly Tyr Ser Ile Ser His Asp  
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His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp  
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Ile Gly His Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro His Leu  
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Gln Asp Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser  
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35

Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr Cys  
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Ala Arg Phe Leu Ala Arg Ile Thr Ala His Asp His Trp Gly Glu Gly  
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Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe  
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55

Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu

EP 2 275 443 A1

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EP 2 275 443 A1

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20	Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys		
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25	Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr		
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30	Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr		
	355	360	365
35	Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu		
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50	Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys		
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55	Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu		

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	His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp 35                      40                      45		
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	Ile Gly Phe Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Ser Leu 50                      55                      60		
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EP 2 275 443 A1

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15	Ala Arg Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr Trp Gly Glu Gly			
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20	Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe			
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25				
30	Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu			
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35	Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp			
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40	Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu			
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50	Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser			
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55	Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro			
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EP 2 275 443 A1

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		245 250 255
20	Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp	
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25	Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn	
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35	Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val	
	290	295 300
40	Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu	
	305	310 315 320
45	Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys	
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5	Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr	355	360	365
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15	Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu	385	390	395
20				400
25	Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys	405	410	415
30	Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu	420	425	430
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Thr Leu Ser Leu Thr Cys Ala Val Ser Gly His Ser Ile Ser His Asp  
 20 25 30

15

His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp  
 35 40 45

20

Ile Gly Phe Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Thr Leu  
 50 55 60

25

Gln Gly Arg Val Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr  
 65 70 75 80

30

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys  
 85 90 95

35

Ala Arg Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr Trp Gly Glu Gly  
 100 105 110

40

Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe  
 115 120 125

45

Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu

50

EP 2 275 443 A1

	130		135		140												
5																	
	Gly	Cys	Leu	Val	Lys	Asp	Tyr	Phe	Pro	Glu	Pro	Val	Thr	Val	Ser	Trp	
	145					150					155					160	
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	Asn	Ser	Gly	Ala	Leu	Thr	Ser	Gly	Val	His	Thr	Phe	Pro	Ala	Val	Leu	
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	Gln	Ser	Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser	
20				180					185					190			
	Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr	Ile	Cys	Asn	Val	Asn	His	Lys	Pro	
25			195					200					205				
	Ser	Asn	Thr	Lys	Val	Asp	Lys	Lys	Val	Glu	Pro	Lys	Ser	Cys	Asp	Lys	
30		210					215					220					
	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Pro	
35																	
	225				230					235						240	
40																	
	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met	Ile	Ser	
45				245					250						255		
	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	His	Glu	Asp	
50				260				265					270				
	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn	
55																	

EP 2 275 443 A1

	275		280		285											
5	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ser	Thr	Tyr	Arg	Val
	290			295							300					
10	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu
15	305					310					315					320
20	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu	Lys
				325						330					335	
25	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr
				340					345					350		
30	Leu	Pro	Pro	Ser	Arg	Asp	Glu	Leu	Thr	Lys	Asn	Gln	Val	Ser	Leu	Thr
		355						360					365			
35	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	Ile	Ala	Val	Glu	Trp	Glu
	370						375					380				
40	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	Thr	Thr	Pro	Pro	Val	Leu
45	385				390					395						400
50	Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	Lys	Leu	Thr	Val	Asp	Lys
				405					410					415		
55	Ser	Arg	Trp	Gln	Gln	Gly	Asn	Val	Phe	Ser	Cys	Ser	Val	Met	His	Glu

5  
420 425 430  
Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly  
435 440 445  
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Lys  
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20 <211> 214  
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35 Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly  
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40 Asp Ser Val Thr Ile Thr Cys Gln Ala Ser Arg Asp Ile Ser Ser His  
20 25 30  
45 Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Glu Leu Leu Ile  
35 40 45  
50 Tyr Tyr Gly Ser His Leu Leu Ser Gly Val Pro Ser Arg Phe Ser Gly  
50 55 60  
55



EP 2 275 443 A1

5	Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Glu Ala	65	70	75	80
10	Glu Asp Ala Ala Thr Tyr Tyr Cys Gly Gln Gly Asn Arg Leu Pro Tyr	85	90	95	
15	Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Glu Arg Thr Val Ala Ala	100	105	110	
20	Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly	115	120	125	
25	Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala	130	135	140	
30	Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln	145	150	155	160
35	Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser	165	170	175	
40	Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr	180	185	190	
45	Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser	195	200	205	
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55					

5 Phe Asn Arg Gly Glu Cys  
210

10 <210> 26  
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<212> PRT  
15 <213> Artificial

<220>  
20 <223> An artificially synthesized peptide sequence

<400> 26

25 Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly  
1 5 10 15

30 Asp Ser Val Thr Ile Thr Cys Gln Ala Ser Arg Asp Ile Ser Ser His  
20 25 30

35 Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Glu Leu Leu Ile  
35 40 45

40 Tyr Tyr Gly Ser His Leu Glu Ser Gly Val Pro Ser Arg Phe Ser Gly  
45 50 55 60

50 Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Glu Ala  
65 70 75 80

55 Glu Asp Ala Ala Thr Tyr Tyr Cys Gly Gln Gly Asn Arg Leu Pro Tyr

**EP 2 275 443 A1**

	85	90	95			
5						
	Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Glu Arg Thr Val Ala Ala					
	100	105	110			
10						
	Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly					
	115	120	125			
15						
	Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala					
	130	135	140			
20						
	Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln					
	145	150	155			
25						
	Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser					
	165	170	175			
30						
	Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr					
	180	185	190			
35						
	Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser					
	195	200	205			
40						
	Phe Asn Arg Gly Glu Cys					
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55	<210>	27				

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<212> PRT

<213> Artificial

<220>

<223> An artificially synthesized peptide sequence

<400> 27

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly  
1 5 10 15

Asp Ser Val Thr Ile Thr Cys Gln Ala Ser Thr Asp Ile Ser Ser His  
20 25 30

Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Glu Leu Leu Ile  
35 40 45

Tyr Tyr Gly Ser His Leu Leu Ser Gly Val Pro Ser Arg Phe Ser Gly  
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Glu Ala  
65 70 75 80

Glu Asp Ala Ala Thr Tyr Tyr Cys Gly Gln Gly Asn Arg Leu Pro Tyr  
85 90 95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Glu Arg Thr Val Ala Ala  
100 105 110

5 Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly  
115 120 125

10 Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala  
130 135 140

15 Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln  
145 150 155 160

20 Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser  
165 170 175

25 Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr  
30 180 185 190

35 Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser  
195 200 205

40 Phe Asn Arg Gly Glu Cys  
210

45 <210> 28

<211> 326

<212> PRT

50 <213> Homo sapiens

<400> 28

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EP 2 275 443 A1

5	Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Cys Ser Arg	1	5	10	15
10	Ser Thr Ser Glu Ser Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr	20	25	30	
15	Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser	35	40	45	
20	Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser	50	55	60	
25	Leu Ser Ser Val Val Thr Val Pro Ser Ser Asn Phe Gly Thr Gln Thr	65	70	75	80
30	Tyr Thr Cys Asn Val Asp His Lys Pro Ser Asn Thr Lys Val Asp Lys	85	90	95	
35	Thr Val Glu Arg Lys Cys Cys Val Glu Cys Pro Pro Cys Pro Ala Pro	100	105	110	
40	Pro Val Ala Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp	115	120	125	
45	Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp	130	135	140	
50					
55					

EP 2 275 443 A1

5	Val Ser His Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly	145	150	155	160
10	Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn	165	170	175	
15	Ser Thr Phe Arg Val Val Ser Val Leu Thr Val Val His Gln Asp Trp	180	185	190	
20	Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu Pro	195	200	205	
25	Ala Pro Ile Glu Lys Thr Ile Ser Lys Thr Lys Gly Gln Pro Arg Glu	210	215	220	
30	Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn	225	230	235	240
35	Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile	245	250	255	
40	Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr	260	265	270	
45	Thr Pro Pro Met Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys	275	280	285	
50					
55					



EP 2 275 443 A1

5           Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys  
               290                               295                               300

10           Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu  
               305                               310                               315                               320

15           Ser Leu Ser Pro Gly Lys  
                                           325

20           <210> 29  
               <211> 324  
               <212> PRT  
 25           <213> Artificial

30           <220>  
               <223> An artificially synthesized peptide sequence

35           <400> 29  
               Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Cys Ser Arg  
               1                               5                               10                               15

40           Ser Thr Ser Glu Ser Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr  
                                   20                               25                               30

45           Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser  
                                   35                               40                               45

50           Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser  
               50                               55                               60

EP 2 275 443 A1

5	Leu Ser Ser Val Val Thr Val Pro Ser Ser Asn Phe Gly Thr Gln Thr			
	65	70	75	80
10	Tyr Thr Cys Asn Val Asp His Lys Pro Ser Asn Thr Lys Val Asp Lys			
		85	90	95
15	Thr Val Glu Arg Lys Cys Cys Val Glu Cys Pro Pro Cys Pro Ala Pro			
		100	105	110
20	Pro Val Ala Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp			
		115	120	125
25	Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp			
		130	135	140
30	Val Ser His Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly			
		145	150	155
35	Val Ser His Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly			
		145	150	155
40	Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn			
		165	170	175
45	Ser Thr Phe Arg Val Val Ser Val Leu Thr Val Val His Gln Asp Trp			
		180	185	190
50	Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu Pro			
		195	200	205

5           Ala Pro Ile Glu Lys Thr Ile Ser Lys Thr Lys Gly Gln Pro Arg Glu  
              210                           215                           220

10           Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn  
              225                           230                           235                           240

15           Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile  
                                  245                           250                           255

20           Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr  
                                  260                           265                           270

25           Thr Pro Pro Met Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys  
                                  275                           280                           285

30           Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys  
                                  290                           295                           300

35           Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu  
              305                           310                           315                           320

40           Ser Leu Ser Pro

45           

50           

55           <210> 30  
              <211> 324

&lt;212&gt; PRT

&lt;213&gt; Artificial

&lt;220&gt;

&lt;223&gt; An artificially synthesized peptide sequence

&lt;400&gt; 30

Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys  
 1 5 10 15

Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr  
 20 25 30

Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser  
 35 40 45

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser  
 50 55 60

Leu Ser Ser Val Val Thr Val Pro Ser Ser Asn Phe Gly Thr Gln Thr  
 65 70 75 80

Tyr Thr Cys Asn Val Asp His Lys Pro Ser Asn Thr Lys Val Asp Lys  
 85 90 95

Thr Val Glu Arg Lys Ser Cys Val Glu Cys Pro Pro Cys Pro Ala Pro  
 100 105 110

EP 2 275 443 A1

5	Pro Val Ala Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp
	115 120 125
10	Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp
	130 135 140
15	Val Ser Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly
	145 150 155 160
20	Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn
	165 170 175
25	Ser Thr Phe Arg Val Val Ser Val Leu Thr Val Val His Gln Asp Trp
	180 185 190
30	Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu Pro
	195 200 205
35	Ala Pro Ile Glu Lys Thr Ile Ser Lys Thr Lys Gly Gln Pro Arg Glu
	210 215 220
40	Pro Gln Val Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn
	225 230 235 240
45	Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile
	245 250 255
50	
55	

EP 2 275 443 A1

Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr  
260 265 270

5

Thr Pro Pro Met Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys  
275 280 285

10

Leu Thr Val Asp Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser Cys  
290 295 300

15

Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu  
305 310 315 320

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25

Ser Leu Ser Pro

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<210> 31

<211> 326

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<212> PRT

<213> Artificial

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<220>

<223> An artificially synthesized peptide sequence

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<400> 31

Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Cys Ser Arg  
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50

Ser Thr Ser Glu Ser Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr  
20 25 30

55

EP 2 275 443 A1

5	Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser	
	35	40 45
10	Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser	
	50	55 60
15	Leu Ser Ser Val Val Thr Val Pro Ser Ser Asn Phe Gly Thr Gln Thr	
	65	70 75 80
20		
	Tyr Thr Cys Asn Val Asp His Lys Pro Ser Asn Thr Lys Val Asp Lys	
25		85 90 95
	Thr Val Glu Arg Lys Cys Cys Val Glu Cys Pro Pro Cys Pro Ala Pro	
30		100 105 110
	Pro Val Ala Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp	
35		115 120 125
40	Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp	
	130	135 140
45	Val Ser His Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly	
	145	150 155 160
50		
	Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn	
55		165 170 175



EP 2 275 443 A1

5	Ser Thr Phe Arg Val Val Ser Val Leu Thr Val Val His Gln Asp Trp	180	185	190
10	Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu Pro	195	200	205
15	Ala Pro Ile Glu Lys Thr Ile Ser Lys Thr Lys Gly Gln Pro Arg Glu	210	215	220
20	Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn	225	230	235
25	Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile	245	250	255
30	Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr	260	265	270
35	Thr Pro Pro Met Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys	275	280	285
40	Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys	290	295	300
45	Ser Val Met His Glu Ala Leu His Ala His Tyr Thr Gln Lys Ser Leu	305	310	315
50				
55				

5 Ser Leu Ser Pro Gly Lys  
 325

10 <210> 32  
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 15 <213> Artificial

<220>  
 20 <223> An artificially synthesized peptide sequence

<400> 32

25 Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys  
 1 5 10 15

30 Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr  
 20 25 30

35 Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser  
 35 40 45

40 Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser  
 45 50 55 60

50 Leu Ser Ser Val Val Thr Val Pro Ser Ser Asn Phe Gly Thr Gln Thr  
 65 70 75 80

55

EP 2 275 443 A1

Tyr Thr Cys Asn Val Asp His Lys Pro Ser Asn Thr Lys Val Asp Lys  
85 90 95

5

Thr Val Glu Arg Lys Ser Cys Val Glu Cys Pro Pro Cys Pro Ala Pro  
100 105 110

10

Pro Val Ala Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp  
115 120 125

15

Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp  
130 135 140

20

Val Ser Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly  
145 150 155 160

25

Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn  
165 170 175

30

Ser Thr Phe Arg Val Val Ser Val Leu Thr Val Val His Gln Asp Trp  
180 185 190

35

Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu Pro  
195 200 205

45

Ala Pro Ile Glu Lys Thr Ile Ser Lys Thr Lys Gly Gln Pro Arg Glu  
210 215 220

50

55

EP 2 275 443 A1

5 Pro Gln Val Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn  
225 230 235 240

10 Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile  
245 250 255

15 Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr  
260 265 270

20 Thr Pro Pro Met Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys  
275 280 285

25 Leu Thr Val Asp Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser Cys  
290 295 300

30 Ser Val Met His Glu Ala Leu His Ala His Tyr Thr Gln Lys Ser Leu  
305 310 315 320

40 Ser Leu Ser Pro

45 <210> 33

<211> 443

<212> PRT

50 <213> Artificial

<220>

<223> An artificially synthesized peptide sequence

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<400> 33

5 Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys Pro Ser Glu  
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10

Thr Leu Ser Leu Thr Cys Ala Val Ser Gly His Ser Ile Ser His Asp

20 25 30

15  
His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp  
35 40 45  
20

Ile Gly Phe Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Ser Leu  
25                50                      55                          60

30            Gln Gly Arg Val Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr  
65                          70                          75                          80

35  
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys  
85 90 95

40

Ala Arg Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr Trp Gly Glu Gly

100 105 110

45

Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe

115 120 125

Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser Thr Ala Ala Leu  
55               130                     135                                 140

EP 2 275 443 A1

5	Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp			
	145	150	155	160
10	Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu			
		165	170	175
15	Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser			
		180	185	190
20	Ser Asn Phe Gly Thr Gln Thr Tyr Thr Cys Asn Val Asp His Lys Pro			
		195	200	205
25	Ser Asn Thr Lys Val Asp Lys Thr Val Glu Arg Lys Cys Cys Val Glu			
		210	215	220
30	Cys Pro Pro Cys Pro Ala Pro Pro Val Ala Gly Pro Ser Val Phe Leu			
		225	230	235
35	Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu			
		245	250	255
40	Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Gln			
		260	265	270
45	Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys			
		275	280	285
50				
55				

5	Pro Arg Glu Glu Gln Phe Asn Ser Thr Phe Arg Val Val Ser Val Leu	290	295	300
10	Thr Val Val His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys	305	310	315
15	Val Ser Asn Lys Gly Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys	325	330	335
20	Thr Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser	340	345	350
25	Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys	355	360	365
30	Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln	370	375	380
35	Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu Asp Ser Asp Gly	385	390	395
40	Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln	405	410	415
45	Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn	420	425	430
50				
55				

5 His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro  
435 440

10 <210> 34  
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15 <213> Artificial

<220>  
20 <223> An artificially synthesized peptide sequence

<400> 34

25 Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys Pro Ser Glu  
1 5 10 15

30 Thr Leu Ser Leu Thr Cys Ala Val Ser Gly His Ser Ile Ser His Asp  
20 25 30

35 His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp  
35 40 45

40 Ile Gly Phe Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Ser Leu  
45 50 55 60

50 Gln Gly Arg Val Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr  
65 70 75 80

55



EP 2 275 443 A1

5	Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys	85	90	95
10	Ala Arg Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr Trp Gly Glu Gly	100	105	110
15	Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe	115	120	125
20	Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu	130	135	140
25	Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp	145	150	155
30				160
35	Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu	165	170	175
40	Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser	180	185	190
45	Ser Asn Phe Gly Thr Gln Thr Tyr Thr Cys Asn Val Asp His Lys Pro	195	200	205
50	Ser Asn Thr Lys Val Asp Lys Thr Val Glu Arg Lys Ser Cys Val Glu	210	215	220
55				

EP 2 275 443 A1

5	Cys Pro Pro Cys Pro Ala Pro Pro Val Ala Gly Pro Ser Val Phe Leu	225	230	235	240
10	Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu	245	250	255	
15	Val Thr Cys Val Val Val Asp Val Ser Gln Glu Asp Pro Glu Val Gln	260	265	270	
20	Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys	275	280	285	
25	Pro Arg Glu Glu Gln Phe Asn Ser Thr Phe Arg Val Val Ser Val Leu	290	295	300	
30	Thr Val Val His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys	305	310	315	320
35	Val Ser Asn Lys Gly Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys	325	330	335	
40	Thr Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser	340	345	350	
45	Gln Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys	355	360	365	
50					
55					

EP 2 275 443 A1

5	Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln	370	375	380	
10	Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu Asp Ser Asp Gly	385	390	395	400
15	Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln	405	410	415	
20	Glu Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn	420	425	430	
25	His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro	435	440		
30					
35	<210> 35				
	<211> 443				
	<212> PRT				
	<213> Artificial				
40	<220>				
	<223> An artificially synthesized peptide sequence				
45	<400> 35				
50	Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys Pro Ser Glu	1	5	10	15
55	Thr Leu Ser Leu Thr Cys Ala Val Ser Gly His Ser Ile Ser His Asp	20	25	30	

5	His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp		
	35	40	45
10	Ile Gly Phe Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Ser Leu		
	50	55	60
15	Gln Gly Arg Val Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr		
20	65	70	75 80
25	Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys		
	85	90	95
30	Ala Arg Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr Trp Gly Glu Gly		
	100	105	110
35	Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe		
	115	120	125
40	Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu		
	130	135	140
45	Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp		
50	145	150	155 160
55	Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu		
	165	170	175

EP 2 275 443 A1

5	Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser	180	185	190
10	Ser Asn Phe Gly Thr Gln Thr Tyr Thr Cys Asn Val Asp His Lys Pro	195	200	205
15	Ser Asn Thr Lys Val Asp Lys Thr Val Glu Arg Lys Ser Cys Val Glu	210	215	220
20	Cys Pro Pro Cys Pro Ala Pro Pro Val Ala Gly Pro Ser Val Phe Leu	225	230	235
25				240
30	Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu	245	250	255
35	Val Thr Cys Val Val Val Asp Val Ser Gln Glu Asp Pro Glu Val Gln	260	265	270
40	Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys	275	280	285
45	Pro Arg Glu Glu Gln Phe Asn Ser Thr Phe Arg Val Val Ser Val Leu	290	295	300
50				
55	Thr Val Val His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys	305	310	315
				320

5	Val Ser Asn Lys Gly Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys	325	330	335
10	Thr Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser	340	345	350
15	Gln Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys	355	360	365
20	Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln	370	375	380
25	Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu Asp Ser Asp Gly	385	390	395
30	Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln	405	410	415
35	Glu Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Ala	420	425	430
40	His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro	435	440	
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55	<210> 36			
	<211> 445			

<212> PRT

<213> Artificial

<220>

<223> An artificially synthesized peptide sequence

<400> 36

Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys Pro Ser Glu  
1 5 10 15

Thr Leu Ser Leu Thr Cys Ala Val Ser Gly Tyr Ser Ile Ser His Asp  
20 25 30

His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp  
35 40 45

Ile Gly His Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro His Leu  
50 55 60

Gln Asp Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser  
65 70 75 80

Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr Cys  
85 90 95

Ala Arg Phe Leu Ala Arg Ile Thr Ala His Asp His Trp Gly Glu Gly  
100 105 110

EP 2 275 443 A1

5	Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe	115	120	125
10	Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser Thr Ala Ala Leu	130	135	140
15	Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp	145	150	155
20	Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu	165	170	175
25	Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser	180	185	190
30	Ser Asn Phe Gly Thr Gln Thr Tyr Thr Cys Asn Val Asp His Lys Pro	195	200	205
35	Ser Asn Thr Lys Val Asp Lys Thr Val Glu Arg Lys Cys Cys Val Glu	210	215	220
40	Cys Pro Pro Cys Pro Ala Pro Pro Val Ala Gly Pro Ser Val Phe Leu	225	230	235
45	Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu	245	250	255

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EP 2 275 443 A1

5	Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Gln	260	265	270
10	Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys	275	280	285
15	Pro Arg Glu Glu Gln Phe Asn Ser Thr Phe Arg Val Val Ser Val Leu	290	295	300
20	Thr Val Val His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys	305	310	315
25				
30	Val Ser Asn Lys Gly Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys	325	330	335
35	Thr Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser	340	345	350
40	Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys	355	360	365
45	Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln	370	375	380
50	Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu Asp Ser Asp Gly	385	390	395
55				400

EP 2 275 443 A1

5 Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln  
405 410 415

10 Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Ala  
420 425 430

15 His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys  
435 440 445

20 <210> 37  
<211> 443  
<212> PRT  
25 <213> Artificial

<220>  
30 <223> An artificially synthesized peptide sequence

<400> 37

35 Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys Pro Ser Glu  
1 5 10 15

40 Thr Leu Ser Leu Thr Cys Ala Val Ser Gly Tyr Ser Ile Ser His Asp  
20 25 30

45 His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp  
35 40 45

50 Ile Gly His Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro His Leu  
55 50 55 60

EP 2 275 443 A1

5	Gln Asp Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser	65	70	75	80
10	Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr Cys	85	90	95	
15	Ala Arg Phe Leu Ala Arg Ile Thr Ala His Asp His Trp Gly Glu Gly	100	105	110	
20	Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe	115	120	125	
25	Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu	130	135	140	
30	Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp	145	150	155	160
35	Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu	165	170	175	
40	Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser	180	185	190	
45	Ser Asn Phe Gly Thr Gln Thr Tyr Thr Cys Asn Val Asp His Lys Pro	195	200	205	
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55					

EP 2 275 443 A1

5	Ser Asn Thr Lys Val Asp Lys Thr Val Glu Arg Lys Ser Cys Val Glu	210	215	220
10	Cys Pro Pro Cys Pro Ala Pro Pro Val Ala Gly Pro Ser Val Phe Leu	225	230	240
15	Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu	245	250	255
20	Val Thr Cys Val Val Val Asp Val Ser Gln Glu Asp Pro Glu Val Gln	260	265	270
25	Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys	275	280	285
30	Pro Arg Glu Glu Gln Phe Asn Ser Thr Phe Arg Val Val Ser Val Leu	290	295	300
35	Thr Val Val His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys	305	310	315
40	Val Ser Asn Lys Gly Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys	325	330	335
45	Thr Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser	340	345	350
50				
55				

EP 2 275 443 A1

5 Gln Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys  
355 360 365

10 Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln  
370 375 380

15 Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu Asp Ser Asp Gly  
385 390 395 400

20 Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln  
405 410 415

25 Glu Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Ala  
420 425 430

30 His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro  
435 440

40 <210> 38  
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<212> PRT  
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45 <220>  
<223> An artificially synthesized peptide sequence

50 <400> 38

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EP 2 275 443 A1

5	Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys Pro Ser Glu	1	5	10	15
10	Thr Leu Ser Leu Thr Cys Ala Val Ser Gly His Ser Ile Ser His Asp	20	25	30	
15	His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp	35	40	45	
20	Ile Gly Phe Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Thr Leu	50	55	60	
25					
30	Gln Gly Arg Val Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr	65	70	75	80
35	Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys	85	90	95	
40	Ala Arg Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr Trp Gly Glu Gly	100	105	110	
45	Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe	115	120	125	
50	Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser Thr Ala Ala Leu	130	135	140	
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EP 2 275 443 A1

5	Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp	145	150	155	160
10	Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu	165	170	175	
15	Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser	180	185	190	
20	Ser Asn Phe Gly Thr Gln Thr Tyr Thr Cys Asn Val Asp His Lys Pro	195	200	205	
25	Ser Asn Thr Lys Val Asp Lys Thr Val Glu Arg Lys Cys Cys Val Glu	210	215	220	
30	Cys Pro Pro Cys Pro Ala Pro Pro Val Ala Gly Pro Ser Val Phe Leu	225	230	235	240
35	Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu	245	250	255	
40	Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Gln	260	265	270	
45	Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys	275	280	285	
50					
55					

EP 2 275 443 A1

5	Pro Arg Glu Glu Gln Phe Asn Ser Thr Phe Arg Val Val Ser Val Leu	290	295	300
10	Thr Val Val His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys	305	310	315 320
15	Val Ser Asn Lys Gly Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys	325	330	335
20	Thr Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser	340	345	350
25	Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys	355	360	365
30	Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln	370	375	380
35	Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu Asp Ser Asp Gly	385	390	395 400
40	Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln	405	410	415
45	Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Ala	420	425	430
50				
55				



His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys  
 435 440 445

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<210> 39  
 <211> 443  
 <212> PRT  
 <213> Artificial

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<220>  
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<400> 39

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Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys Pro Ser Glu  
 1 5 10 15

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Thr Leu Ser Leu Thr Cys Ala Val Ser Gly His Ser Ile Ser His Asp  
 20 25 30

30

His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp  
 35 40 45

35

Ile Gly Phe Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Thr Leu  
 50 55 60

40

Gln Gly Arg Val Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr  
 65 70 75 80

45

50

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys  
 85 90 95

55

EP 2 275 443 A1

5	Ala Arg Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr Trp Gly Glu Gly	100	105	110
10	Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe	115	120	125
15	Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu	130	135	140
20	Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp	145	150	155
25				160
30	Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu	165	170	175
35	Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser	180	185	190
40	Ser Asn Phe Gly Thr Gln Thr Tyr Thr Cys Asn Val Asp His Lys Pro	195	200	205
45	Ser Asn Thr Lys Val Asp Lys Thr Val Glu Arg Lys Ser Cys Val Glu	210	215	220
50	Cys Pro Pro Cys Pro Ala Pro Pro Val Ala Gly Pro Ser Val Phe Leu	225	230	235
55				240

EP 2 275 443 A1

5	Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu	245	250	255
10	Val Thr Cys Val Val Val Asp Val Ser Gln Glu Asp Pro Glu Val Gln	260	265	270
15	Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys	275	280	285
20	Pro Arg Glu Glu Gln Phe Asn Ser Thr Phe Arg Val Val Ser Val Leu	290	295	300
25	Thr Val Val His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys	305	310	315
30				320
35	Val Ser Asn Lys Gly Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys	325	330	335
40	Thr Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser	340	345	350
45	Gln Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys	355	360	365
50	Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln	370	375	380
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5 Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu Asp Ser Asp Gly  
385 390 395 400

10 Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln  
405 410 415

15 Glu Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Ala  
420 425 430

20 His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro  
435 440

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<211> 6  
<212> PRT  
<213> Artificial

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<220>  
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<400> 40

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His Asp His Ala Trp Ser  
1 5

50

<210> 41  
<211> 16  
<212> PRT  
<213> Artificial

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&lt;220&gt;

&lt;223&gt; An artificially synthesized peptide sequence

&lt;400&gt; 41

His	Ile	Ser	Tyr	Ser	Gly	Ile	Thr	Asn	Tyr	Asn	Pro	His	Leu	Gln	Asp
1				5					10				15		

&lt;210&gt; 42

&lt;211&gt; 16

&lt;212&gt; PRT

&lt;213&gt; Artificial

&lt;220&gt;

&lt;223&gt; An artificially synthesized peptide sequence

&lt;400&gt; 42

Phe	Ile	Ser	Tyr	Ser	Gly	Ile	Thr	Asn	Tyr	Asn	Pro	Ser	Leu	Gln	Gly
1				5					10				15		

&lt;210&gt; 43

&lt;211&gt; 16

&lt;212&gt; PRT

&lt;213&gt; Artificial

&lt;220&gt;

&lt;223&gt; An artificially synthesized peptide sequence

&lt;400&gt; 43

Phe	Ile	Ser	Tyr	Ser	Gly	Ile	Thr	Asn	Tyr	Asn	Pro	Thr	Leu	Gln	Gly
1				5					10				15		

5           <210> 44  
            <211> 10  
            <212> PRT  
            <213> Artificial

10           <220>  
            <223> An artificially synthesized peptide sequence

15           <400> 44

20           Phe Leu Ala Arg Ile Thr Ala His Asp His  
            1                           5                           10

25           <210> 45  
            <211> 10  
            <212> PRT  
            <213> Artificial

30           <220>  
            <223> An artificially synthesized peptide sequence

35           <400> 45

40           Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr  
            1                           5                           10

45           <210> 46  
            <211> 30  
            <212> PRT

50           <213> Artificial

55           <220>

<223> An artificially synthesized peptide sequence

5 <400> 46

10 Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys Pro Ser Glu  
1 5 10 15

15 Thr Leu Ser Leu Thr Cys Ala Val Ser Gly Tyr Ser Ile Ser  
20 25 30

20 <210> 47

<211> 30

<212> PRT

25 <213> Artificial

<220>

30 <223> An artificially synthesized peptide sequence

<400> 47

35 Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys Pro Ser Glu  
1 5 10 15

40 Thr Leu Ser Leu Thr Cys Ala Val Ser Gly His Ser Ile Ser  
20 25 30

45 <210> 48

<211> 14

50 <212> PRT

<213> Artificial

55 <220>

<223> An artificially synthesized peptide sequence

<400> 48

Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp Ile Gly

1 5 10

<210> 49

<211> 32

<212> PRT

<213> Artificial

<220>

<223> An artificially synthesized peptide sequence

<400> 49

Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser Leu Lys

1 5 10 15

Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Ala Tyr Tyr Cys Ala Arg

20 25 30

<210> 50

<211> 32

<212> PRT

<213> Artificial

<220>

<223> An artificially synthesized peptide sequence

<400> 50



Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser Leu Lys  
1 5 10 15

Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr Cys Ala Arg  
20 25 30

<210> 51

<211> 32

<212> PRT

<213> Artificial

<220>

<223> An artificially synthesized peptide sequence

<400> 51

Arg Val Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr Leu Gln  
1 5 10 15

Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg  
20 25 30

<210> 52

<211> 11

<212> PRT

<213> Artificial

<220>

<223> An artificially synthesized peptide sequence

<400> 52

5 Trp Gly Glu Gly Thr Leu Val Thr Val Ser Ser  
1 5 10

10 <210> 53  
<211> 11  
<212> PRT  
<213> Artificial

15 <220>  
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20 <400> 53

25 Gln Ala Ser Arg Asp Ile Ser Ser His Leu Asn  
1 5 10

30 <210> 54  
<211> 11  
<212> PRT  
<213> Artificial

35 <220>  
<223> An artificially synthesized peptide sequence

40 <400> 54

45 Gln Ala Ser Thr Asp Ile Ser Ser His Leu Asn  
1 5 10

50 <210> 55  
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55 <213> Artificial

5 <220>  
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10 <400> 55

Tyr Gly Ser His Leu Leu Ser  
 1 5

15

<210> 56  
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 <212> PRT  
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20

25 <220>  
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30 <400> 56

Tyr Gly Ser His Leu Glu Ser  
 1 5

35

<210> 57  
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 <213> Artificial

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45 <220>  
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50 <400> 57

Gly Gln Gly Asn Arg Leu Pro Tyr Thr  
 1 5

55

5           <210> 58  
           <211> 23  
           <212> PRT  
           <213> Artificial  
  
 10           <220>  
           <223> An artificially synthesized peptide sequence  
  
 15           <400> 58  
  
 20           Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly  
               1                               5                               10                               15  
  
 25           Asp Ser Val Thr Ile Thr Cys  
                               20  
  
 30           <210> 59  
           <211> 15  
           <212> PRT  
 35           <213> Artificial  
  
           <220>  
 40           <223> An artificially synthesized peptide sequence  
  
           <400> 59  
  
 45           Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Glu Leu Leu Ile Tyr  
               1                               5                               10                               15  
  
 50           <210> 60  
           <211> 32  
 55

<212> PRT  
<213> Artificial

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<220>  
<223> An artificially synthesized peptide sequence

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<400> 60

15

Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr  
1 5 10 15

20

Phe Thr Ile Ser Ser Leu Glu Ala Glu Asp Ala Ala Thr Tyr Tyr Cys  
20 25 30

25

<210> 61  
<211> 10  
<212> PRT  
<213> Artificial

30

<220>  
<223> An artificially synthesized peptide sequence

35

<400> 61

40

Phe Gly Gln Gly Thr Lys Val Glu Ile Glu  
1 5 10

45

<210> 62  
<211> 449  
<212> PRT  
<213> Artificial

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55

<220>

<223> An artificially synthesized peptide sequence

<400> 62

Glu Val Gln Leu Val Glu Ser Gly Gly Lys Leu Leu Lys Pro Gly Gly  
1 5 10 15

Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Phe  
20 25 30

Ala Met Ser Trp Phe Arg Gln Ser Pro Glu Lys Arg Leu Glu Trp Val  
35 40 45

Ala Glu Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro Asp Thr Val  
50 55 60

Thr Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr  
65 70 75 80

Leu Glu Met Ser Ser Leu Arg Ser Glu Asp Thr Ala Met Tyr Tyr Cys  
85 90 95

Ala Arg Gly Leu Trp Gly Tyr Tyr Ala Leu Asp Tyr Trp Gly Gln Gly  
100 105 110

Thr Ser Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe  
115 120 125

EP 2 275 443 A1

5	Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu	130	135	140
10	Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp	145	150	155 160
15	Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu	165	170	175
20	Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser	180	185	190
25	Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro	195	200	205
30	Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys	210	215	220
35	Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro	225	230	235 240
40	Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser	245	250	255
45	Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp	260	265	270
50				
55				

EP 2 275 443 A1

Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn  
275 280 285

5

Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val  
290 295 300

10

Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu  
305 310 315 320

15

Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys  
325 330 335

20

Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr  
340 345 350

25

Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr  
355 360 365

30

Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu  
370 375 380

35

Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu  
385 390 395 400

40

Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys  
405 410 415

45

50



EP 2 275 443 A1

Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu  
420 425 430

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Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly  
435 440 445

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Lys

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<210> 63

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Glu Lys Val Thr Met Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met  
20 25 30

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Tyr Trp Tyr Gln Gln Lys Pro Gly Ser Ser Pro Arg Leu Leu Ile Tyr  
35 40 45

45

Asp Thr Ser Asn Leu Ala Ser Gly Val Pro Val Arg Phe Ser Gly Ser  
50 55 60

50

55

5	Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Arg Met Glu Ala Glu			
	65	70	75	80
10	Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Gly Tyr Pro Tyr Thr			
		85	90	95
15	Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg Thr Val Ala Ala Pro			
		100	105	110
20	Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr			
25		115	120	125
30	Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys			
		130	135	140
35	Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu			
	145	150	155	160
40	Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser			
		165	170	175
45	Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala			
50		180	185	190
55	Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe			
	195	200	205	

Asn Arg Gly Glu Cys

210

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&lt;211&gt; 449

&lt;212&gt; PRT

&lt;213&gt; Artificial

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&lt;220&gt;

&lt;223&gt; An artificially synthesized peptide sequence

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&lt;400&gt; 64

Glu Val Gln Leu Val Glu Ser Gly Gly Lys Leu Leu Lys Pro Gly Gly

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15

Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser His

30

20

25

30

Ala Met Ser Trp Phe Arg Gln Ser Pro Glu Lys Arg Leu Glu Trp Val

35

35

40

45

Ala Glu Ile Ser Ser Gly Gly Ser Tyr Thr Tyr His Pro His Thr Val

40

50

55

60

45

Thr Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr

50

65

70

75

80

55

EP 2 275 443 A1

5	Leu Glu Met Ser Ser Leu Arg Ser Glu Asp Thr Ala Met Tyr Tyr Cys	85	90	95
10	Ala Arg Gly Leu Trp Gly His Tyr Ala Leu Asp Tyr Trp Gly Gln Gly	100	105	110
15	Thr Ser Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe	115	120	125
20	Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu	130	135	140
25	Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp	145	150	155
30	Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu	165	170	175
35	Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser	180	185	190
40	Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro	195	200	205
45	Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys	210	215	220
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55				

EP 2 275 443 A1

5	Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro	225	230	235	240
10	Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser	245	250	255	
15	Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp	260	265	270	
20	Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn	275	280	285	
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30	Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val	290	295	300	
35	Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu	305	310	315	320
40	Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys	325	330	335	
45	Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr	340	345	350	
50	Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr	355	360	365	
55					

EP 2 275 443 A1

5	Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu
	370 375 380
10	Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu
	385 390 395 400
15	Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys
	405 410 415
20	Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu
	420 425 430
25	Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly
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5	Glu Lys Val Thr Met Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met	
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10	Tyr Trp Tyr Gln Gln Lys Pro Gly Ser Ser Pro Arg Leu Leu Ile Tyr	
	35	40 45
15	Asp Thr Ser Asn His Ala Ser Gly Val Pro Val Arg Phe Ser Gly Ser	
	50	55 60
20	Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Arg Met Glu Ala Glu	
	65	70 75 80
25	Asp Ala Ala Thr Tyr Tyr Cys Gln His Trp Ser Gly His Pro Tyr Thr	
	85	90 95
30	Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg Thr Val Ala Ala Pro	
	100	105 110
35	Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr	
	115	120 125
40	Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys	
	130	135 140
45	Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu	
	145	150 155 160

5

Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser  
                                   165                                  170                                  175

10

Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala  
                                   180                                  185                                  190

15

Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe  
                                   195                                  200                                  205

20

Asn Arg Gly Glu Cys  
                                   210

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30

&lt;210&gt; 66

&lt;211&gt; 213

&lt;212&gt; PRT

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&lt;213&gt; Artificial

&lt;220&gt;

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&lt;223&gt; An artificially synthesized peptide sequence

&lt;400&gt; 66

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Gln Ile Val Leu Ile Gln Ser Pro Ala Ile Met Ser Ala Ser Pro Gly  
   1                                  5                                  10                                  15

50

Glu Lys Val Thr Met Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met  
                                   20                                  25                                  30

55



EP 2 275 443 A1

5	Tyr Trp Tyr Gln Gln Lys Pro Gly Ser Ser Pro Arg Leu Leu Ile Tyr	35	40	45
10	Asp Thr Ser His His Ala Ser Gly Val Pro Val Arg Phe Ser Gly Ser	50	55	60
15	Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Arg Met Glu Ala Glu	65	70	75
20	Asp Ala Ala Thr Tyr Tyr Cys Gln His Trp Ser Gly His Pro Tyr Thr	85	90	95
25	Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg Thr Val Ala Ala Pro	100	105	110
30	Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr	115	120	125
35	Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys	130	135	140
40	Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu	145	150	155
45	Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser	165	170	175
50				
55				

Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala  
180 185 190

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Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe  
195 200 205

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Asn Arg Gly Glu Cys  
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Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala  
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Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Gly Tyr  
20 25 30

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Tyr Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met  
35 40 45

45

Gly Trp Ile Asn Pro Asn Ser Gly Gly Thr Asn Tyr Ala Gln Lys Phe  
50 55 60

50

55

EP 2 275 443 A1

5	Gln Gly Arg Val Thr Met Thr Arg Asp Thr Ser Ile Ser Thr Ala Tyr	65	70	75	80
10	Met Glu Leu Ser Arg Leu Arg Ser Asp Asp Thr Ala Val Tyr Tyr Cys	85	90	95	
15	Ala Arg Glu Asp Val Val Pro Ala Ala Met Ser Phe Tyr Tyr Gly Met	100	105	110	
20	Asp Val Trp Gly Arg Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr	115	120	125	
25	Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser	130	135	140	
30	Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu	145	150	155	160
35	Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His	165	170	175	
40	Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser	180	185	190	
45	Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys	195	200	205	
50					
55					

EP 2 275 443 A1

5	Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu	210	215	220
10	Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro	225	230	235 240
15	Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys	245	250	255
20	Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val	260	265	270
25	Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp	275	280	285
30	Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr	290	295	300
35	Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp	305	310	315 320
40	Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu	325	330	335
45	Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg	340	345	350
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55				

5	Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys	355	360	365	
10	Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp	370	375	380	
15	Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys	385	390	395	400
20	Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser	405	410	415	
25	Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser	420	425	430	
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35	Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser	435	440	445	
40	Leu Ser Leu Ser Pro Gly Lys Gly	450	455		
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50	<213> Artificial				
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Leu Pro Val Leu Thr Gln Pro Pro Ser Val Ser Val Ala Pro Gly Gln  
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Thr Ala Arg Ile Thr Cys Gly Gly Asn Asn Ile Gly Ser Lys Thr Val  
20 25 30

Tyr Trp Tyr Gln Gln Glu Pro Gly Gln Ala Pro Val Leu Val Val Tyr  
35 40 45

Asp Asp Thr Asp Arg Pro Ala Gly Ile Pro Glu Arg Phe Ser Gly Ala  
50 55 60

Asn Ser Gly Asn Thr Ala Thr Leu Thr Ile Ser Arg Val Glu Ala Gly  
65 70 75 80

Asp Glu Ala Asp Tyr Tyr Cys Gln Val Trp Asp Ser Ser Thr Asp His  
85 90 95

Gly Val Phe Gly Gly Gly Thr Lys Leu Thr Val Leu Gly Gln Pro Lys  
100 105 110

Ala Asn Pro Thr Val Thr Leu Phe Pro Pro Ser Ser Glu Glu Leu Gln  
115 120 125

EP 2 275 443 A1

Ala Asn Lys Ala Thr Leu Val Cys Leu Ile Ser Asp Phe Tyr Pro Gly  
130 135 140

5

Ala Val Thr Val Ala Trp Lys Ala Asp Gly Ser Pro Val Lys Ala Gly  
145 150 155 160

10

Val Glu Thr Thr Lys Pro Ser Lys Gln Ser Asn Asn Lys Tyr Ala Ala  
165 170 175

15

Ser Ser Tyr Leu Ser Leu Thr Pro Glu Gln Trp Lys Ser His Arg Ser  
180 185 190

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Tyr Ser Cys Gln Val Thr His Glu Gly Ser Thr Val Glu Lys Thr Val  
195 200 205

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30

Ala Pro Thr Glu Cys Ser  
210

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<210> 69  
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<220>  
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<400> 69

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala  
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55

5	Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Gly Tyr		
		20	25 30
10	His Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met		
		35	40 45
15			
	Gly Trp Ile Asn Pro Asn Ser Gly Gly Thr Asn Tyr Ala Gln Lys Phe		
		50	55 60
20			
	Gln Gly Arg Val Thr Met Thr Arg Asp Thr Ser Ile Ser Thr Ala Tyr		
25		65	70 75 80
	Met Glu Leu Ser Arg Leu Arg Ser Asp Asp Thr Ala Val Tyr Tyr Cys		
30		85	90 95
35	Ala Arg Glu Asp Val Val Pro Ala Ala Met Ser Phe Tyr Tyr Gly Met		
		100	105 110
40			
	Asp Val Trp Gly Arg Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr		
		115	120 125
45			
	Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser		
		130	135 140
50			
	Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu		
55		145	150 155 160



5	Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His		
		165	170 175
10	Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser		
		180	185 190
15	Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys		
		195	200 205
20	Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu		
25		210	215 220
30	Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro		
		225	230 235 240
35	Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys		
		245	250 255
40	Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val		
		260	265 270
45	Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp		
		275	280 285
50	Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr		
55		290	295 300

EP 2 275 443 A1

5	Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp			
	305	310	315	320
10	Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu			
		325	330	335
15	Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg			
		340	345	350
20	Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys			
25		355	360	365
30	Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp			
	370	375	380	
35	Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys			
	385	390	395	400
40	Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser			
		405	410	415
45	Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser			
50		420	425	430
55	Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser			
	435	440	445	

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10           <210> 70  
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30           Thr Leu Ser Leu Thr Cys Ala Val Ser Gly Tyr Ser Ile Ser Asp Asp  
                           20                       25                       30

35           Gln Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp  
                           35                       40                       45

40           Ile Gly Tyr Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Ser Leu  
                   50                       55                       60

45           Lys Gly Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser  
               65                       70                       75                       80

50           

55

EP 2 275 443 A1

	Leu	Lys	Leu	Ser	Ser	Val	Thr	Ala	Ala	Asp	Thr	Ala	Ala	Tyr	Tyr	Cys
					85					90					95	
5																
	Ala	Arg	Ser	Leu	Ala	Arg	Thr	Thr	Ala	Met	Asp	Tyr	Trp	Gly	Glu	Gly
10				100					105					110		
	Thr	Leu	Val	Thr	Val	Ser	Ser	Ala	Ser	Thr	Lys	Gly	Pro	Ser	Val	Phe
15			115					120					125			
	Pro	Leu	Ala	Pro	Ser	Ser	Lys	Ser	Thr	Ser	Gly	Gly	Thr	Ala	Ala	Leu
20			130				135					140				
25	Gly	Cys	Leu	Val	Lys	Asp	Tyr	Phe	Pro	Glu	Pro	Val	Thr	Val	Ser	Trp
	145					150					155					160
30																
	Asn	Ser	Gly	Ala	Leu	Thr	Ser	Gly	Val	His	Thr	Phe	Pro	Ala	Val	Leu
35					165					170					175	
	Gln	Ser	Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser
40				180					185					190		
	Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr	Ile	Cys	Asn	Val	Asn	His	Lys	Pro
45			195					200					205			
50	Ser	Asn	Thr	Lys	Val	Asp	Lys	Lys	Val	Glu	Pro	Lys	Ser	Cys	Asp	Lys
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EP 2 275 443 A1

Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro  
225 230 235 240

5

Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser  
245 250 255

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Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp  
260 265 270

15

Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn  
275 280 285

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Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val  
290 295 300

25

Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu  
305 310 315 320

30

Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys  
325 330 335

35

Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr  
340 345 350

40

Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr  
355 360 365

50

55

EP 2 275 443 A1

Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu  
370 375 380

5

Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu  
385 390 395 400

10

Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys  
405 410 415

15

Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu  
420 425 430

20

Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly  
435 440 445

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Lys

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<151> 2008-04-11

<150> JP 2008-247713  
<151> 2008-09-26

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<151> 2009-03-19

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35 Thr Leu Ser Leu Thr Cys Ala Val Ser Gly Tyr Ser Ile Ser Asp Asp  
20 25 30

His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp  
35 40 45

40 Ile Gly Tyr Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Ser Leu  
50 55 60

45 Lys Gly Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser  
65 70 75 80

Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Ala Tyr Tyr Cys  
85 90 95

50 Ala Arg Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr Trp Gly Glu Gly  
100 105 110

55 Thr Leu Val Thr Val Ser Ser

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                   20                   25                   30  
  
 20       Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Glu Leu Leu Ile  
                   35                   40                   45  
  
           Tyr Tyr Gly Ser Glu Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly  
 25           50                   55                   60  
  
           Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Glu Ala  
           65                   70                   75                   80  
  
 30       Glu Asp Ala Ala Thr Tyr Tyr Cys Gly Gln Gly Asn Arg Leu Pro Tyr  
                   85                   90                   95  
  
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                   20                   25                   30  
  
           His Ala His Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp  
 55           35                   40                   45



# EP 2 275 443 A1

5 Ile Gly Tyr Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Ser Leu  
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Lys Gly Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser  
 65 70 75 80

10 Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Ala Tyr Tyr Cys  
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15 Ala Arg Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr Trp Gly Glu Gly  
 100 105 110

Thr Leu Val Thr Val Ser Ser  
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 1 5 10 15

35 Thr Leu Ser Leu Thr Cys Ala Val Ser Gly His Ser Ile Ser His His  
 20 25 30

His Ala His Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp  
 35 40 45

40 Ile Gly Tyr Ile Ser Tyr Ser Gly Ile Thr His Tyr Asn Pro His Leu  
 50 55 60

45 Lys Gly Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser  
 65 70 75 80

Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Ala Tyr Tyr Cys  
 85 90 95

50 Ala Arg Val Leu Ala Arg Ile Thr Ala Met Asp His Trp Gly Glu Gly  
 100 105 110

55 Thr Leu Val Thr Val Ser Ser

115

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                   20                   25                   30  
  
 20       His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp  
                   35                   40                   45  
  
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 25           50                   55                   60  
  
           Lys Gly Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser  
           65                   70                   75                   80  
  
 30       Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Ala Tyr Tyr Cys  
                   85                   90                   95  
  
           Ala Arg Phe Leu Ala Arg Ile Thr Ala His Asp His Trp Gly Glu Gly  
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 55           20                   25                   30

# EP 2 275 443 A1

5 Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Glu Leu Leu Ile  
 35 40 45  
 Tyr Tyr Gly Ser His Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly  
 50 55 60  
 10 Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Glu Ala  
 65 70 75 80  
 15 Glu Asp Ala Ala Thr Tyr Tyr Cys Gly Gln Gly Asn Arg Leu Pro Tyr  
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 Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Glu  
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 35 Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Glu Leu Leu Ile  
 35 40 45  
 40 Tyr Tyr Gly Ser His Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly  
 50 55 60  
 45 Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Glu Ala  
 65 70 75 80  
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# EP 2 275 443 A1

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15 Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Glu Leu Leu Ile  
35 40 45

20 Tyr Tyr Gly Ser His Leu His His Gly Val Pro Ser Arg Phe Ser Gly  
50 55 60

25 Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Glu Ala  
65 70 75 80

Glu Asp Ala Ala Thr Tyr Tyr Cys Gly Gln Gly His Arg Leu Pro Tyr  
85 90 95

30 Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Glu  
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50 His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Arg Gly Leu Glu Trp  
35 40 45

55 Ile Gly Tyr Ile Ser Tyr Ser Gly Ile Thr Thr Tyr Asn Pro Ser Leu  
50 55 60

# EP 2 275 443 A1

5	Lys 65	Ser	Arg	Val	Thr	Met 70	Leu	Arg	Asp	Thr	Ser 75	Lys	Asn	Gln	Phe	Ser 80
	Leu	Arg	Leu	Ser	Ser 85	Val	Thr	Ala	Ala	Asp 90	Thr	Ala	Val	Tyr	Tyr 95	Cys
10	Ala	Arg	Ser	Leu 100	Ala	Arg	Thr	Thr	Ala 105	Met	Asp	Tyr	Trp	Gly 110	Gln	Gly
15	Ser	Leu	Val 115	Thr	Val	Ser	Ser	Ala 120	Ser	Thr	Lys	Gly	Pro 125	Ser	Val	Phe
	Pro	Leu	Ala	Pro	Ser	Ser	Lys 135	Ser	Thr	Ser	Gly	Gly 140	Thr	Ala	Ala	Leu
20	Gly 145	Cys	Leu	Val	Lys	Asp 150	Tyr	Phe	Pro	Glu	Pro 155	Val	Thr	Val	Ser	Trp 160
25	Asn	Ser	Gly	Ala	Leu 165	Thr	Ser	Gly	Val	His 170	Thr	Phe	Pro	Ala	Val 175	Leu
	Gln	Ser	Ser	Gly 180	Leu	Tyr	Ser	Leu	Ser 185	Ser	Val	Val	Thr	Val 190	Pro	Ser
30	Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr 200	Ile	Cys	Asn	Val	Asn 205	His	Lys	Pro
35	Ser	Asn	Thr	Lys	Val	Asp	Lys 215	Lys	Val	Glu	Pro	Lys 220	Ser	Cys	Asp	Lys
40	Thr 225	His	Thr	Cys	Pro	Pro 230	Cys	Pro	Ala	Pro	Glu 235	Leu	Leu	Gly	Gly	Pro 240
	Ser	Val	Phe	Leu	Phe 245	Pro	Pro	Lys	Pro	Lys 250	Asp	Thr	Leu	Met	Ile	Ser 255
45	Arg	Thr	Pro	Glu 260	Val	Thr	Cys	Val	Val 265	Val	Asp	Val	Ser	His 270	Glu	Asp
50	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr 280	Val	Asp	Gly	Val	Glu 285	Val	His	Asn
55	Ala	Lys	Thr	Lys	Pro	Arg	Glu 295	Glu	Gln	Tyr	Asn	Ser 300	Thr	Tyr	Arg	Val

# EP 2 275 443 A1

Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu  
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 Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys  
 325 330 335  
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 Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr  
 340 345 350  
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 Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr  
 355 360 365  
 Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu  
 370 375 380  
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 Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu  
 385 390 395 400  
 25  
 Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys  
 405 410 415  
 Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu  
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 Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile  
 35 40 45  
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 Tyr Tyr Thr Ser Arg Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly  
 50 55 60

# EP 2 275 443 A1

5 Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Gln Pro  
 65 70 75 80  
 Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Gly Asn Thr Leu Pro Tyr  
 85 90 95  
 10 Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala  
 100 105 110  
 15 Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly  
 115 120 125  
 Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala  
 130 135 140  
 20 Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln  
 145 150 155 160  
 25 Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser  
 165 170 175  
 Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr  
 180 185 190  
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 195 200 205  
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 55 His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp

# EP 2 275 443 A1

	35	40	45
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10	Lys Gly Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser 65 70 75 80		
	Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Ala Tyr Tyr Cys 85 90 95		
15	Ala Arg Val Leu Ala Arg Ile Thr Ala Met Asp Tyr Trp Gly Glu Gly 100 105 110		
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25	<210> 12 <211> 107 <212> PRT <213> Artificial		
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40	Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Glu Leu Leu Ile 35 40 45		
	Tyr Tyr Gly Ser Glu Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly 50 55 60		
45	Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Glu Ala 65 70 75 80		
50	Glu Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Gly Asn Ser Leu Pro Tyr 85 90 95		
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# EP 2 275 443 A1

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 tgcgccgtgt ctggctactc aattagcgac gatcatgcct ggagctgggt tcgccagcca 180  
 15 cctggagaag gtcttgagtg gattggatac attagttata gtggaatcac aaactataat 240  
 ccatctctca aaggcagagt gacaatatcg agagacacca gcaagaacca gttcagcctg 300  
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 40 aagtgcagg tctccaacaa agccctcca gccccatcg agaaaaccat ctccaaagcc 1080  
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 aagaaccagg tcagcctgac ctgcctggtc aaaggcttct atcccagcga catcgccgtg 1200  
 45 gagtgggaga gcaatgggca gccggagaac aactacaaga ccacgcctcc cgtgctggac 1260  
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 10 ggaaaggctc cagagctgct gatctactac ggctccgaac tgcactctgg tgtgccaagc 180  
 agattcagcg gtagcggtag cggtaccgac ttcaccttca ccatcagcag cctcgaggca 240  
 15 gaggacgccg ctacctacta ctgcgggcag ggtaaccggc ttccatacac gttcggccaa 300  
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 tctgccagga gacagcgtga ctctgacctg cccaggggga gagccggaag acaatgccac 180  
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45 tgtcgggagc aagctcagca aaactcagac ctttcagggt tgtggaatct tgcagcctga 660  
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50 ctggcaagac cccactcct ggaactcatc tttctacaga ctacggtttg agctcagata 780  
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55 gttcgggcaa ggcgagtgga gcgagtggag cccggaggcc atgggcacgc cttggacaga 960

# EP 2 275 443 A1

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25 His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp  
 35 40 45

Ile Gly His Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro His Leu  
 50 55 60

30 Gln Asp Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser  
 65 70 75 80

35 Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Ala Tyr Tyr Cys  
 85 90 95

40 Ala Arg Phe Leu Ala Arg Ile Thr Ala His Asp His Trp Gly Glu Gly  
 100 105 110

Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe  
 115 120 125

45 Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu  
 130 135 140

50 Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp  
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Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu  
 165 170 175

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# EP 2 275 443 A1

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			195					200					205			
10	Ser	Asn	Thr	Lys	Val	Asp	Lys	Lys	Val	Glu	Pro	Lys	Ser	Cys	Asp	Lys
		210					215					220				
	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Pro
	225					230					235					240
15	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met	Ile	Ser
					245					250					255	
20	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	His	Glu	Asp
				260					265					270		
25	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn
			275					280					285			
	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ser	Thr	Tyr	Arg	Val
		290					295					300				
30	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu
	305					310					315					320
35	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu	Lys
					325					330					335	
	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr
				340					345					350		
40	Leu	Pro	Pro	Ser	Arg	Asp	Glu	Leu	Thr	Lys	Asn	Gln	Val	Ser	Leu	Thr
			355					360					365			
45	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	Ile	Ala	Val	Glu	Trp	Glu
		370					375					380				
	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	Thr	Thr	Pro	Pro	Val	Leu
	385					390					395					400
50	Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	Lys	Leu	Thr	Val	Asp	Lys
					405					410					415	
55	Ser	Arg	Trp	Gln	Gln	Gly	Asn	Val	Phe	Ser	Cys	Ser	Val	Met	His	Glu

# EP 2 275 443 A1

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	His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp		
	35	40	45
30			
	Ile Gly His Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro His Leu		
	50	55	60
	Gln Asp Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser		
35		70	75 80
	Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr Cys		
	85	90	95
40			
	Ala Arg Phe Leu Ala Arg Ile Thr Ala His Asp His Trp Gly Glu Gly		
	100	105	110
	Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe		
45		120	125
	Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu		
50		135	140
	Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp		
	145	150	155 160
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# EP 2 275 443 A1

	Asn	Ser	Gly	Ala	Leu	Thr	Ser	Gly	Val	His	Thr	Phe	Pro	Ala	Val	Leu	
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10	Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr	Ile	Cys	Asn	Val	Asn	His	Lys	Pro	
			195					200					205				
15	Ser	Asn	Thr	Lys	Val	Asp	Lys	Lys	Val	Glu	Pro	Lys	Ser	Cys	Asp	Lys	
		210					215					220					
20	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Pro	
	225					230					235					240	
25	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met	Ile	Ser	
					245					250					255		
30	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	His	Glu	Asp	
				260					265					270			
35	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn	
			275					280					285				
40	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ser	Thr	Tyr	Arg	Val	
		290					295					300					
45	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu	
	305					310				315						320	
50	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu	Lys	
					325					330					335		
55	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr	
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60	Leu	Pro	Pro	Ser	Arg	Asp	Glu	Leu	Thr	Lys	Asn	Gln	Val	Ser	Leu	Thr	
			355					360					365				
65	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	Ile	Ala	Val	Glu	Trp	Glu	
		370					375					380					
70	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	Thr	Thr	Pro	Pro	Val	Leu	
	385					390					395					400	
75	Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	Lys	Leu	Thr	Val	Asp	Lys	

# EP 2 275 443 A1

	405	410	415
5	Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu 420 425 430		
10	Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly 435 440 445		
	Lys		
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30	Thr Leu Ser Leu Thr Cys Ala Val Ser Gly His Ser Ile Ser His Asp 20 25 30		
35	His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp 35 40 45		
40	Ile Gly Phe Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Ser Leu 50 55 60		
	Gln Gly Arg Val Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr 65 70 75 80		
45	Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys 85 90 95		
50	Ala Arg Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr Trp Gly Glu Gly 100 105 110		
	Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe 115 120 125		
55	Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu 130 135 140		



# EP 2 275 443 A1

	Gly	Cys	Leu	Val	Lys	Asp	Tyr	Phe	Pro	Glu	Pro	Val	Thr	Val	Ser	Trp
	145					150					155					160
5	Asn	Ser	Gly	Ala	Leu	Thr	Ser	Gly	Val	His	Thr	Phe	Pro	Ala	Val	Leu
					165					170					175	
10	Gln	Ser	Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser
				180					185					190		
15	Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr	Ile	Cys	Asn	Val	Asn	His	Lys	Pro
			195					200					205			
20	Ser	Asn	Thr	Lys	Val	Asp	Lys	Lys	Val	Glu	Pro	Lys	Ser	Cys	Asp	Lys
		210					215					220				
25	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Pro
	225					230					235					240
30	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met	Ile	Ser
					245					250					255	
35	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	His	Glu	Asp
				260					265					270		
40	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn
			275					280					285			
45	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ser	Thr	Tyr	Arg	Val
		290					295					300				
50	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu
	305					310					315					320
55	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu	Lys
					325					330					335	
60	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr
				340					345					350		
65	Leu	Pro	Pro	Ser	Arg	Asp	Glu	Leu	Thr	Lys	Asn	Gln	Val	Ser	Leu	Thr
			355					360					365			
70	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	Ile	Ala	Val	Glu	Trp	Glu
		370					375					380				
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EP 2 275 443 A1

	385		390		395		400									
5	Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	Lys	Leu	Thr	Val	Asp	Lys
					405					410					415	
	Ser	Arg	Trp	Gln	Gln	Gly	Asn	Val	Phe	Ser	Cys	Ser	Val	Met	His	Glu
10				420					425					430		
	Ala	Leu	His	Asn	His	Tyr	Thr	Gln	Lys	Ser	Leu	Ser	Leu	Ser	Pro	Gly
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15	Lys															
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				20					25					30		
	His	Ala	Trp	Ser	Trp	Val	Arg	Gln	Pro	Pro	Gly	Glu	Gly	Leu	Glu	Trp
35			35					40					45			
	Ile	Gly	Phe	Ile	Ser	Tyr	Ser	Gly	Ile	Thr	Asn	Tyr	Asn	Pro	Thr	Leu
40		50					55					60				
	Gln	Gly	Arg	Val	Thr	Ile	Ser	Arg	Asp	Asn	Ser	Lys	Asn	Thr	Leu	Tyr
	65					70					75				80	
45	Leu	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp	Thr	Ala	Val	Tyr	Tyr	Cys
					85					90					95	
	Ala	Arg	Ser	Leu	Ala	Arg	Thr	Thr	Ala	Met	Asp	Tyr	Trp	Gly	Glu	Gly
50				100					105					110		
	Thr	Leu	Val	Thr	Val	Ser	Ser	Ala	Ser	Thr	Lys	Gly	Pro	Ser	Val	Phe
				115				120					125			
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# EP 2 275 443 A1

	Pro	Leu	Ala	Pro	Ser	Ser	Lys	Ser	Thr	Ser	Gly	Gly	Thr	Ala	Ala	Leu	
		130					135					140					
5	Gly	Cys	Leu	Val	Lys	Asp	Tyr	Phe	Pro	Glu	Pro	Val	Thr	Val	Ser	Trp	
	145					150					155					160	
	Asn	Ser	Gly	Ala	Leu	Thr	Ser	Gly	Val	His	Thr	Phe	Pro	Ala	Val	Leu	
10					165					170					175		
	Gln	Ser	Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser	
				180					185					190			
15	Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr	Ile	Cys	Asn	Val	Asn	His	Lys	Pro	
			195					200					205				
	Ser	Asn	Thr	Lys	Val	Asp	Lys	Lys	Val	Glu	Pro	Lys	Ser	Cys	Asp	Lys	
20		210					215					220					
	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Pro	
	225					230					235					240	
25																	
	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met	Ile	Ser	
					245					250					255		
30	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	His	Glu	Asp	
				260					265					270			
	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn	
35			275					280					285				
	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ser	Thr	Tyr	Arg	Val	
		290					295					300					
40	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu	
	305					310					315					320	
	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu	Lys	
45					325					330					335		
	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr	
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50	Leu	Pro	Pro	Ser	Arg	Asp	Glu	Leu	Thr	Lys	Asn	Gln	Val	Ser	Leu	Thr	
			355					360					365				
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EP 2 275 443 A1

	370	375	380
5	Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu 385 390 395 400		
10	Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys 405 410 415		
15	Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu 420 425 430		
20	Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly 435 440 445		
	Lys		
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40	Asp Ser Val Thr Ile Thr Cys Gln Ala Ser Arg Asp Ile Ser Ser His 20 25 30		
	Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Glu Leu Leu Ile 35 40 45		
45	Tyr Tyr Gly Ser His Leu Leu Ser Gly Val Pro Ser Arg Phe Ser Gly 50 55 60		
50	Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Glu Ala 65 70 75 80		
	Glu Asp Ala Ala Thr Tyr Tyr Cys Gly Gln Gly Asn Arg Leu Pro Tyr 85 90 95		
55	Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Glu Arg Thr Val Ala Ala 100 105 110		

# EP 2 275 443 A1

Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly  
115 120 125

5 Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala  
130 135 140

10 Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln  
145 150 155 160

Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser  
165 170 175

15 Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr  
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20 Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser  
195 200 205

Phe Asn Arg Gly Glu Cys  
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20 25 30

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35 40 45

45 Tyr Tyr Gly Ser His Leu Glu Ser Gly Val Pro Ser Arg Phe Ser Gly  
50 55 60

50 Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Glu Ala  
65 70 75 80

Glu Asp Ala Ala Thr Tyr Tyr Cys Gly Gln Gly Asn Arg Leu Pro Tyr  
85 90 95

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# EP 2 275 443 A1

	Thr	Phe	Gly	Gln	Gly	Thr	Lys	Val	Glu	Ile	Glu	Arg	Thr	Val	Ala	Ala	
				100					105					110			
5																	
	Pro	Ser	Val	Phe	Ile	Phe	Pro	Pro	Ser	Asp	Glu	Gln	Leu	Lys	Ser	Gly	
			115					120					125				
10																	
	Thr	Ala	Ser	Val	Val	Cys	Leu	Leu	Asn	Asn	Phe	Tyr	Pro	Arg	Glu	Ala	
		130					135					140					
15																	
	Lys	Val	Gln	Trp	Lys	Val	Asp	Asn	Ala	Leu	Gln	Ser	Gly	Asn	Ser	Gln	
	145					150					155					160	
	Glu	Ser	Val	Thr	Glu	Gln	Asp	Ser	Lys	Asp	Ser	Thr	Tyr	Ser	Leu	Ser	
					165					170					175		
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	Ser	Thr	Leu	Thr	Leu	Ser	Lys	Ala	Asp	Tyr	Glu	Lys	His	Lys	Val	Tyr	
				180					185					190			
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	Ala	Cys	Glu	Val	Thr	His	Gln	Gly	Leu	Ser	Ser	Pro	Val	Thr	Lys	Ser	
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	Asp	Ser	Val	Thr	Ile	Thr	Cys	Gln	Ala	Ser	Thr	Asp	Ile	Ser	Ser	His	
				20					25					30			
	Leu	Asn	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Lys	Ala	Pro	Glu	Leu	Leu	Ile	
			35					40					45				
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	Tyr	Tyr	Gly	Ser	His	Leu	Leu	Ser	Gly	Val	Pro	Ser	Arg	Phe	Ser	Gly	
		50					55					60					
55																	
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	65					70					75				80		

# EP 2 275 443 A1

5           Glu Asp Ala Ala Thr Tyr Tyr Cys Gly Gln Gly Asn Arg Leu Pro Tyr  
                                   85                                   90                                   95  
  
           Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Glu Arg Thr Val Ala Ala  
                           100                                   105                                   110  
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           Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly  
                           115                                   120                                   125  
  
           Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala  
                   130                                   135                                   140  
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           Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln  
           145                                   150                                   155                                   160  
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           Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser  
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           Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr  
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           Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser  
                           35                                   40                                   45  
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           Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser  
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EP 2 275 443 A1

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5	Tyr	Thr	Cys	Asn	Val	Asp	His	Lys	Pro	Ser	Asn	Thr	Lys	Val	Asp	Lys	85	90	95	
10	Thr	Val	Glu	Arg	Lys	Cys	Cys	Val	Glu	Cys	Pro	Pro	Cys	Pro	Ala	Pro	100	105	110	
	Pro	Val	Ala	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	115	120	125	
15	Thr	Leu	Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp	130	135	140	
20	Val	Ser	His	Glu	Asp	Pro	Glu	Val	Gln	Phe	Asn	Trp	Tyr	Val	Asp	Gly	145	150	155	160
25	Val	Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Phe	Asn	165	170	175	
	Ser	Thr	Phe	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Val	His	Gln	Asp	Trp	180	185	190	
30	Leu	Asn	Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Gly	Leu	Pro	195	200	205	
35	Ala	Pro	Ile	Glu	Lys	Thr	Ile	Ser	Lys	Thr	Lys	Gly	Gln	Pro	Arg	Glu	210	215	220	
40	Pro	Gln	Val	Tyr	Thr	Leu	Pro	Pro	Ser	Arg	Glu	Glu	Met	Thr	Lys	Asn	225	230	235	240
	Gln	Val	Ser	Leu	Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	Ile	245	250	255	
45	Ala	Val	Glu	Trp	Glu	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	Thr	260	265	270	
50	Thr	Pro	Pro	Met	Leu	Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	Lys	275	280	285	
	Leu	Thr	Val	Asp	Lys	Ser	Arg	Trp	Gln	Gln	Gly	Asn	Val	Phe	Ser	Cys	290	295	300	
55	Ser	Val	Met	His	Glu	Ala	Leu	His	Asn	His	Tyr	Thr	Gln	Lys	Ser	Leu				



# EP 2 275 443 A1

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						10	
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							Pro
							Cys
							Ser
							Arg
							15
20	Ser	Thr	Ser	Glu	Ser	Thr	Ala
				20			
						Ala	Leu
						25	
						Gly	Cys
							Leu
							Val
							Lys
							Asp
							Tyr
							30
	Phe	Pro	Glu	Pro	Val	Thr	Val
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						40	
						Asn	Ser
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							Ala
							Leu
							Thr
							Ser
							45
	Gly	Val	His	Thr	Phe	Pro	Ala
		50				55	
							Leu
							Gln
							Ser
							Ser
							Gly
							Leu
							Tyr
							Ser
							60
30	Leu	Ser	Ser	Val	Val	Thr	Val
	65					70	
							Pro
							Ser
							Ser
							Asn
							Phe
							Gly
							Thr
							Gln
							Thr
							80
35	Tyr	Thr	Cys	Asn	Val	Asp	His
				85			
							Lys
							Pro
							Ser
							Asn
							Thr
							Lys
							Val
							Asp
							Lys
							95
	Thr	Val	Glu	Arg	Lys	Cys	Cys
			100				
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							Glu
							105
							Cys
							Pro
							Pro
							Cys
							Pro
							Ala
							Pro
							110
40	Pro	Val	Ala	Gly	Pro	Ser	Val
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							Phe
							120
							Leu
							Phe
							Pro
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							Lys
							Pro
							Lys
							Asp
							125
45	Thr	Leu	Met	Ile	Ser	Arg	Thr
		130					
							Pro
							Glu
							Val
							Thr
							Cys
							140
							Val
							Val
							Val
							Asp
							Gly
							160
50	Val	Ser	His	Glu	Asp	Pro	Glu
	145					150	
							Val
							Gln
							Phe
							Asn
							Trp
							Tyr
							Val
							Asp
							Gly
							165
	Val	Glu	Val	His	Asn	Ala	Lys
							Thr
							Lys
							Pro
							Arg
							Glu
							Glu
							Gln
							Phe
							Asn
							175
55							

# EP 2 275 443 A1

Ser Thr Phe Arg Val Val Ser Val Leu Thr Val Val His Gln Asp Trp  
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5 Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu Pro  
195 200 205

10 Ala Pro Ile Glu Lys Thr Ile Ser Lys Thr Lys Gly Gln Pro Arg Glu  
210 215 220

Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn  
225 230 235 240

15 Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile  
245 250 255

20 Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr  
260 265 270

Thr Pro Pro Met Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys  
275 280 285

25 Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys  
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35 40 45

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EP 2 275 443 A1

	Gly	Val	His	Thr	Phe	Pro	Ala	Val	Leu	Gln	Ser	Ser	Gly	Leu	Tyr	Ser
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5																
	Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser	Ser	Asn	Phe	Gly	Thr	Gln	Thr
	65					70					75					80
10	Tyr	Thr	Cys	Asn	Val	Asp	His	Lys	Pro	Ser	Asn	Thr	Lys	Val	Asp	Lys
					85					90					95	
	Thr	Val	Glu	Arg	Lys	Ser	Cys	Val	Glu	Cys	Pro	Pro	Cys	Pro	Ala	Pro
15				100					105					110		
	Pro	Val	Ala	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp
			115					120					125			
20	Thr	Leu	Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp
	130						135					140				
	Val	Ser	Gln	Glu	Asp	Pro	Glu	Val	Gln	Phe	Asn	Trp	Tyr	Val	Asp	Gly
25	145					150					155					160
	Val	Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Phe	Asn
					165					170					175	
30																
	Ser	Thr	Phe	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Val	His	Gln	Asp	Trp
				180					185					190		
35	Leu	Asn	Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Gly	Leu	Pro
			195					200					205			
	Ala	Pro	Ile	Glu	Lys	Thr	Ile	Ser	Lys	Thr	Lys	Gly	Gln	Pro	Arg	Glu
40		210					215					220				
	Pro	Gln	Val	Tyr	Thr	Leu	Pro	Pro	Ser	Gln	Glu	Glu	Met	Thr	Lys	Asn
	225					230					235					240
45	Gln	Val	Ser	Leu	Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	Ile
					245					250					255	
	Ala	Val	Glu	Trp	Glu	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	Thr
50				260					265					270		
	Thr	Pro	Pro	Met	Leu	Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	Lys
			275					280					285			
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# EP 2 275 443 A1

Leu Thr Val Asp Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser Cys  
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 30 35 40 45

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser  
 50 55 60

35 Leu Ser Ser Val Val Thr Val Pro Ser Ser Asn Phe Gly Thr Gln Thr  
 65 70 75 80

Tyr Thr Cys Asn Val Asp His Lys Pro Ser Asn Thr Lys Val Asp Lys  
 40 85 90 95

Thr Val Glu Arg Lys Cys Cys Val Glu Cys Pro Pro Cys Pro Ala Pro  
 45 100 105 110

Pro Val Ala Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp  
 115 120 125

50 Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp  
 130 135 140

Val Ser His Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly  
 55 145 150 155 160

# EP 2 275 443 A1

Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn  
 165 170 175  
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 Ser Thr Phe Arg Val Val Ser Val Leu Thr Val Val His Gln Asp Trp  
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 Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu Pro  
 195 200 205  
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 Ala Pro Ile Glu Lys Thr Ile Ser Lys Thr Lys Gly Gln Pro Arg Glu  
 210 215 220  
 Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn  
 225 230 235 240  
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 Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile  
 245 250 255  
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 Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr  
 260 265 270  
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 Thr Pro Pro Met Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys  
 275 280 285  
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 Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys  
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EP 2 275 443 A1

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			35					40					45			
	Gly	Val	His	Thr	Phe	Pro	Ala	Val	Leu	Gln	Ser	Ser	Gly	Leu	Tyr	Ser
		50					55					60				
10	Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser	Ser	Asn	Phe	Gly	Thr	Gln	Thr
	65					70					75					80
	Tyr	Thr	Cys	Asn	Val	Asp	His	Lys	Pro	Ser	Asn	Thr	Lys	Val	Asp	Lys
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	Thr	Val	Glu	Arg	Lys	Ser	Cys	Val	Glu	Cys	Pro	Pro	Cys	Pro	Ala	Pro
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	Pro	Val	Ala	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp
			115					120					125			
25	Thr	Leu	Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp
		130					135					140				
	Val	Ser	Gln	Glu	Asp	Pro	Glu	Val	Gln	Phe	Asn	Trp	Tyr	Val	Asp	Gly
30						150					155					160
	Val	Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Phe	Asn
				165						170					175	
35	Ser	Thr	Phe	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Val	His	Gln	Asp	Trp
				180					185					190		
	Leu	Asn	Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Gly	Leu	Pro
40			195					200					205			
	Ala	Pro	Ile	Glu	Lys	Thr	Ile	Ser	Lys	Thr	Lys	Gly	Gln	Pro	Arg	Glu
45			210				215					220				
	Pro	Gln	Val	Tyr	Thr	Leu	Pro	Pro	Ser	Gln	Glu	Glu	Met	Thr	Lys	Asn
	225					230					235					240
50	Gln	Val	Ser	Leu	Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	Ile
				245						250					255	
	Ala	Val	Glu	Trp	Glu	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	Thr
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# EP 2 275 443 A1

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 Leu Thr Val Asp Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser Cys  
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 His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp  
 35 35 40 45  
 Ile Gly Phe Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Ser Leu  
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 40 Gln Gly Arg Val Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr  
 65 70 75 80  
 Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys  
 45 85 90 95  
 Ala Arg Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr Trp Gly Glu Gly  
 100 105 110  
 50 Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe  
 115 120 125  
 55 Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser Thr Ala Ala Leu  
 130 135 140

EP 2 275 443 A1

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	Asn	Ser	Gly	Ala	Leu	Thr	Ser	Gly	Val	His 170	Thr	Phe	Pro	Ala	Val	Leu 175
10	Gln	Ser	Ser	Gly 180	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser 190
15	Ser	Asn	Phe 195	Gly	Thr	Gln	Thr	Tyr 200	Thr	Cys	Asn	Val	Asp 205	His	Lys	Pro
	Ser	Asn 210	Thr	Lys	Val	Asp	Lys 215	Thr	Val	Glu	Arg	Lys 220	Cys	Cys	Val	Glu
20	Cys 225	Pro	Pro	Cys	Pro	Ala 230	Pro	Pro	Val	Ala	Gly 235	Pro	Ser	Val	Phe	Leu 240
25	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met 250	Ile	Ser	Arg	Thr	Pro	Glu 255
30	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	His	Glu	Asp	Pro	Glu	Val	Gln 270
	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn	Ala 285	Lys	Thr	Lys
35	Pro	Arg	Glu	Glu	Gln	Phe	Asn 295	Ser	Thr	Phe	Arg	Val 300	Val	Ser	Val	Leu
40	Thr 305	Val	Val	His	Gln	Asp 310	Trp	Leu	Asn	Gly	Lys 315	Glu	Tyr	Lys	Cys	Lys 320
	Val	Ser	Asn	Lys	Gly 325	Leu	Pro	Ala	Pro	Ile 330	Glu	Lys	Thr	Ile	Ser	Lys 335
45	Thr	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln 345	Val	Tyr	Thr	Leu	Pro	Pro	Ser 350
50	Arg	Glu	Glu	Met	Thr	Lys	Asn	Gln 360	Val	Ser	Leu	Thr	Cys 365	Leu	Val	Lys
55	Gly 370	Phe	Tyr	Pro	Ser	Asp	Ile 375	Ala	Val	Glu	Trp	Glu 380	Ser	Asn	Gly	Gln



# EP 2 275 443 A1

Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu Asp Ser Asp Gly  
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 Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln  
 405 410 415  
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 Ile Gly Phe Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Ser Leu  
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 Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys  
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 Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe  
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 Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu  
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EP 2 275 443 A1

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10	Gln	Ser	Ser	Gly 180	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser 190
15	Ser	Asn	Phe 195	Gly	Thr	Gln	Thr	Tyr 200	Thr	Cys	Asn	Val	Asp 205	His	Lys	Pro
	Ser	Asn 210	Thr	Lys	Val	Asp	Lys 215	Thr	Val	Glu	Arg	Lys 220	Ser	Cys	Val	Glu
20	Cys 225	Pro	Pro	Cys	Pro	Ala 230	Pro	Pro	Val	Ala	Gly 235	Pro	Ser	Val	Phe	Leu 240
25	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met 250	Ile	Ser	Arg	Thr	Pro	Glu 255
30	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	Gln	Glu	Asp	Pro	Glu	Val	Gln 270
	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn	Ala 285	Lys	Thr	Lys
35	Pro	Arg	Glu	Glu	Gln	Phe	Asn 295	Ser	Thr	Phe	Arg	Val 300	Val	Ser	Val	Leu
40	Thr 305	Val	Val	His	Gln	Asp 310	Trp	Leu	Asn	Gly	Lys 315	Glu	Tyr	Lys	Cys	Lys 320
	Val	Ser	Asn	Lys	Gly 325	Leu	Pro	Ala	Pro	Ile 330	Glu	Lys	Thr	Ile	Ser	Lys 335
45	Thr	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln 345	Val	Tyr	Thr	Leu	Pro	Pro	Ser 350
50	Gln	Glu	Glu	Met	Thr	Lys	Asn	Gln 360	Val	Ser	Leu	Thr	Cys 365	Leu	Val	Lys
55	Gly 370	Phe	Tyr	Pro	Ser	Asp	Ile 375	Ala	Val	Glu	Trp	Glu 380	Ser	Asn	Gly	Gln

# EP 2 275 443 A1

Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu Asp Ser Asp Gly  
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 Ile Gly Phe Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Ser Leu  
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 Gln Gly Arg Val Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr  
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 Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe  
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EP 2 275 443 A1

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10	Gln	Ser	Ser	Gly 180	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser 190
15	Ser	Asn	Phe 195	Gly	Thr	Gln	Thr	Tyr 200	Thr	Cys	Asn	Val	Asp 205	His	Lys	Pro
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30	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	Gln	Glu	Asp	Pro	Glu	Val	Gln 270
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35	Pro	Arg 290	Glu	Glu	Gln	Phe	Asn 295	Ser	Thr	Phe	Arg	Val	Val	Ser	Val	Leu
40	Thr 305	Val	Val	His	Gln	Asp 310	Trp	Leu	Asn	Gly	Lys 315	Glu	Tyr	Lys	Cys	Lys 320
	Val	Ser	Asn	Lys	Gly 325	Leu	Pro	Ala	Pro	Ile 330	Glu	Lys	Thr	Ile	Ser	Lys 335
45	Thr	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln 345	Val	Tyr	Thr	Leu	Pro	Pro	Ser 350
50	Gln	Glu	Glu	Met	Thr	Lys	Asn	Gln 360	Val	Ser	Leu	Thr	Cys 365	Leu	Val	Lys
55	Gly 370	Phe	Tyr	Pro	Ser	Asp	Ile 375	Ala	Val	Glu	Trp	Glu 380	Ser	Asn	Gly	Gln

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 Ile Gly His Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro His Leu  
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 Gln Asp Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser  
 65 70 75 80  
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 Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr Cys  
 85 90 95  
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 Ala Arg Phe Leu Ala Arg Ile Thr Ala His Asp His Trp Gly Glu Gly  
 100 105 110  
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 Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe  
 115 120 125  
 Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser Thr Ala Ala Leu  
 130 135 140

EP 2 275 443 A1

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10	Gln	Ser	Ser	Gly 180	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser 190
15	Ser	Asn	Phe 195	Gly	Thr	Gln	Thr	Tyr 200	Thr	Cys	Asn	Val	Asp 205	His	Lys	Pro
	Ser	Asn 210	Thr	Lys	Val	Asp	Lys 215	Thr	Val	Glu	Arg	Lys 220	Cys	Cys	Val	Glu
20	Cys 225	Pro	Pro	Cys	Pro	Ala 230	Pro	Pro	Val	Ala	Gly 235	Pro	Ser	Val	Phe	Leu 240
25	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met 250	Ile	Ser	Arg	Thr	Pro	Glu 255
30	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	His	Glu	Asp	Pro	Glu	Val	Gln 270
	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn	Ala 285	Lys	Thr	Lys
35	Pro	Arg	Glu	Glu	Gln	Phe	Asn 295	Ser	Thr	Phe	Arg	Val 300	Val	Ser	Val	Leu
40	Thr 305	Val	Val	His	Gln	Asp 310	Trp	Leu	Asn	Gly	Lys 315	Glu	Tyr	Lys	Cys	Lys 320
	Val	Ser	Asn	Lys	Gly 325	Leu	Pro	Ala	Pro	Ile 330	Glu	Lys	Thr	Ile	Ser	Lys 335
45	Thr	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln 345	Val	Tyr	Thr	Leu	Pro	Pro	Ser 350
50	Arg	Glu	Glu	Met	Thr	Lys	Asn	Gln 360	Val	Ser	Leu	Thr	Cys 365	Leu	Val	Lys
55	Gly 370	Phe	Tyr	Pro	Ser	Asp	Ile 375	Ala	Val	Glu	Trp	Glu 380	Ser	Asn	Gly	Gln

# EP 2 275 443 A1

5 Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu Asp Ser Asp Gly  
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 Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln  
 405 410 415  
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 Ile Gly His Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro His Leu  
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 40 Gln Asp Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser  
 65 70 75 80  
 45 Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr Cys  
 85 90 95  
 Ala Arg Phe Leu Ala Arg Ile Thr Ala His Asp His Trp Gly Glu Gly  
 100 105 110  
 50 Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe  
 115 120 125  
 55 Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu  
 130 135 140

EP 2 275 443 A1

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10	Gln	Ser	Ser	Gly 180	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser 190
15	Ser	Asn	Phe 195	Gly	Thr	Gln	Thr	Tyr 200	Thr	Cys	Asn	Val	Asp 205	His	Lys	Pro
	Ser	Asn 210	Thr	Lys	Val	Asp	Lys 215	Thr	Val	Glu	Arg	Lys 220	Ser	Cys	Val	Glu
20	Cys 225	Pro	Pro	Cys	Pro	Ala 230	Pro	Pro	Val	Ala	Gly 235	Pro	Ser	Val	Phe	Leu 240
25	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met 250	Ile	Ser	Arg	Thr	Pro	Glu 255
30	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	Gln	Glu	Asp	Pro	Glu	Val	Gln 270
	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn	Ala 285	Lys	Thr	Lys
35	Pro	Arg 290	Glu	Glu	Gln	Phe	Asn 295	Ser	Thr	Phe	Arg	Val	Val	Ser	Val	Leu
40	Thr 305	Val	Val	His	Gln	Asp 310	Trp	Leu	Asn	Gly	Lys 315	Glu	Tyr	Lys	Cys	Lys 320
	Val	Ser	Asn	Lys	Gly 325	Leu	Pro	Ala	Pro	Ile 330	Glu	Lys	Thr	Ile	Ser	Lys 335
45	Thr	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln 345	Val	Tyr	Thr	Leu	Pro	Pro	Ser 350
50	Gln	Glu	Glu	Met	Thr	Lys	Asn	Gln 360	Val	Ser	Leu	Thr	Cys 365	Leu	Val	Lys
55	Gly 370	Phe	Tyr	Pro	Ser	Asp	Ile 375	Ala	Val	Glu	Trp	Glu 380	Ser	Asn	Gly	Gln



# EP 2 275 443 A1

Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu Asp Ser Asp Gly  
 385 390 395 400

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Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln  
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His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp  
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Ile Gly Phe Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Thr Leu  
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Gln Gly Arg Val Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr  
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55

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys  
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60

Ala Arg Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr Trp Gly Glu Gly  
 100 105 110

65

Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe  
 115 120 125

70

Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser Thr Ala Ala Leu  
 130 135 140

EP 2 275 443 A1

5	Gly 145	Cys	Leu	Val	Lys	Asp 150	Tyr	Phe	Pro	Glu	Pro 155	Val	Thr	Val	Ser	Trp 160
	Asn	Ser	Gly	Ala	Leu	Thr	Ser	Gly	Val	His 170	Thr	Phe	Pro	Ala	Val	Leu 175
10	Gln	Ser	Ser	Gly 180	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser 190
15	Ser	Asn	Phe 195	Gly	Thr	Gln	Thr	Tyr 200	Thr	Cys	Asn	Val	Asp 205	His	Lys	Pro
	Ser	Asn 210	Thr	Lys	Val	Asp	Lys 215	Thr	Val	Glu	Arg	Lys 220	Cys	Cys	Val	Glu
20	Cys 225	Pro	Pro	Cys	Pro	Ala 230	Pro	Pro	Val	Ala	Gly 235	Pro	Ser	Val	Phe	Leu 240
25	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met 250	Ile	Ser	Arg	Thr	Pro	Glu 255
30	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	His	Glu	Asp	Pro	Glu	Val	Gln 270
	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn	Ala 285	Lys	Thr	Lys
35	Pro	Arg 290	Glu	Glu	Gln	Phe	Asn 295	Ser	Thr	Phe	Arg	Val 300	Val	Ser	Val	Leu
40	Thr 305	Val	Val	His	Gln	Asp 310	Trp	Leu	Asn	Gly	Lys 315	Glu	Tyr	Lys	Cys	Lys 320
	Val	Ser	Asn	Lys	Gly 325	Leu	Pro	Ala	Pro	Ile 330	Glu	Lys	Thr	Ile	Ser	Lys 335
45	Thr	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln 345	Val	Tyr	Thr	Leu	Pro	Pro	Ser 350
50	Arg	Glu	Glu	Met	Thr	Lys	Asn	Gln 360	Val	Ser	Leu	Thr	Cys 365	Leu	Val	Lys
55	Gly 370	Phe	Tyr	Pro	Ser	Asp	Ile 375	Ala	Val	Glu	Trp	Glu 380	Ser	Asn	Gly	Gln

EP 2 275 443 A1

Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu Asp Ser Asp Gly  
385 390 395 400

5 Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln  
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10 Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Ala  
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30 Thr Leu Ser Leu Thr Cys Ala Val Ser Gly His Ser Ile Ser His Asp  
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35 His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp  
35 40 45

Ile Gly Phe Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Thr Leu  
50 55 60

40 Gln Gly Arg Val Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr  
65 70 75 80

45 Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys  
85 90 95

Ala Arg Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr Trp Gly Glu Gly  
100 105 110

50 Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe  
115 120 125

55 Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu  
130 135 140

EP 2 275 443 A1

5	Gly 145	Cys	Leu	Val	Lys	Asp 150	Tyr	Phe	Pro	Glu	Pro 155	Val	Thr	Val	Ser	Trp 160
	Asn	Ser	Gly	Ala	Leu	Thr	Ser	Gly	Val	His 170	Thr	Phe	Pro	Ala	Val	Leu 175
10	Gln	Ser	Ser	Gly 180	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser 190
15	Ser	Asn	Phe 195	Gly	Thr	Gln	Thr	Tyr 200	Thr	Cys	Asn	Val	Asp 205	His	Lys	Pro
	Ser	Asn 210	Thr	Lys	Val	Asp	Lys 215	Thr	Val	Glu	Arg	Lys 220	Ser	Cys	Val	Glu
20	Cys 225	Pro	Pro	Cys	Pro	Ala 230	Pro	Pro	Val	Ala	Gly 235	Pro	Ser	Val	Phe	Leu 240
25	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met 250	Ile	Ser	Arg	Thr	Pro	Glu 255
30	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	Gln	Glu	Asp	Pro	Glu	Val	Gln 270
	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn	Ala 285	Lys	Thr	Lys
35	Pro	Arg 290	Glu	Glu	Gln	Phe	Asn 295	Ser	Thr	Phe	Arg	Val	Val	Ser	Val	Leu
40	Thr 305	Val	Val	His	Gln	Asp 310	Trp	Leu	Asn	Gly	Lys 315	Glu	Tyr	Lys	Cys	Lys 320
	Val	Ser	Asn	Lys	Gly 325	Leu	Pro	Ala	Pro	Ile 330	Glu	Lys	Thr	Ile	Ser	Lys 335
45	Thr	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln 345	Val	Tyr	Thr	Leu	Pro	Pro	Ser 350
50	Gln	Glu	Glu	Met	Thr	Lys	Asn	Gln 360	Val	Ser	Leu	Thr	Cys 365	Leu	Val	Lys
55	Gly 370	Phe	Tyr	Pro	Ser	Asp	Ile 375	Ala	Val	Glu	Trp	Glu 380	Ser	Asn	Gly	Gln

# EP 2 275 443 A1

5 Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu Asp Ser Asp Gly  
 385 390 395 400

Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln  
 405 410 415

10 Glu Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Ala  
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15 His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro  
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Phe Leu Ala Arg Ile Thr Ala His Asp His  
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Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys Pro Ser Glu  
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50 Thr Leu Ser Leu Thr Cys Ala Val Ser Gly Tyr Ser Ile Ser  
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 Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser Leu Lys  
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 40 Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Ala Tyr Tyr Cys Ala Arg  
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 Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser Leu Lys  
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# EP 2 275 443 A1

Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr Cys Ala Arg  
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Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg  
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Gln Ala Ser Arg Asp Ile Ser Ser His Leu Asn  
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EP 2 275 443 A1

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 Phe Thr Ile Ser Ser Leu Glu Ala Glu Asp Ala Ala Thr Tyr Tyr Cys  
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 Glu Val Gln Leu Val Glu Ser Gly Gly Lys Leu Leu Lys Pro Gly Gly  
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# EP 2 275 443 A1

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			20						25					30		
	Ala	Met	Ser	Trp	Phe	Arg	Gln	Ser	Pro	Glu	Lys	Arg	Leu	Glu	Trp	Val
			35					40					45			
10	Ala	Glu	Ile	Ser	Ser	Gly	Gly	Ser	Tyr	Thr	Tyr	Tyr	Pro	Asp	Thr	Val
		50					55					60				
	Thr	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	Lys	Asn	Thr	Leu	Tyr
15	65					70					75				80	
	Leu	Glu	Met	Ser	Ser	Leu	Arg	Ser	Glu	Asp	Thr	Ala	Met	Tyr	Tyr	Cys
					85					90				95		
20	Ala	Arg	Gly	Leu	Trp	Gly	Tyr	Tyr	Ala	Leu	Asp	Tyr	Trp	Gly	Gln	Gly
				100					105					110		
	Thr	Ser	Val	Thr	Val	Ser	Ser	Ala	Ser	Thr	Lys	Gly	Pro	Ser	Val	Phe
25			115					120					125			
	Pro	Leu	Ala	Pro	Ser	Ser	Lys	Ser	Thr	Ser	Gly	Gly	Thr	Ala	Ala	Leu
		130					135					140				
30	Gly	Cys	Leu	Val	Lys	Asp	Tyr	Phe	Pro	Glu	Pro	Val	Thr	Val	Ser	Trp
	145					150					155					160
	Asn	Ser	Gly	Ala	Leu	Thr	Ser	Gly	Val	His	Thr	Phe	Pro	Ala	Val	Leu
35					165					170					175	
	Gln	Ser	Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser
40				180					185					190		
	Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr	Ile	Cys	Asn	Val	Asn	His	Lys	Pro
			195					200					205			
45	Ser	Asn	Thr	Lys	Val	Asp	Lys	Lys	Val	Glu	Pro	Lys	Ser	Cys	Asp	Lys
		210					215					220				
	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Pro
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	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met	Ile	Ser
				245						250					255	
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# EP 2 275 443 A1

	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	His	Glu	Asp
				260					265					270		
5	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn
			275					280					285			
10	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ser	Thr	Tyr	Arg	Val
		290					295					300				
	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu
	305					310					315					320
15	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu	Lys
					325					330					335	
20	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr
				340					345					350		
	Leu	Pro	Pro	Ser	Arg	Asp	Glu	Leu	Thr	Lys	Asn	Gln	Val	Ser	Leu	Thr
25			355					360					365			
	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	Ile	Ala	Val	Glu	Trp	Glu
		370					375					380				
30	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	Thr	Thr	Pro	Pro	Val	Leu
	385					390					395					400
	Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	Lys	Leu	Thr	Val	Asp	Lys
35					405					410					415	
	Ser	Arg	Trp	Gln	Gln	Gly	Asn	Val	Phe	Ser	Cys	Ser	Val	Met	His	Glu
				420					425					430		
40	Ala	Leu	His	Asn	His	Tyr	Thr	Gln	Lys	Ser	Leu	Ser	Leu	Ser	Pro	Gly
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# EP 2 275 443 A1

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	Glu	Lys	Val	Thr	Met	Thr	Cys	Ser	Ala	Ser	Ser	Ser	Val	Ser	Tyr	Met
				20					25					30		
10	Tyr	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Ser	Ser	Pro	Arg	Leu	Leu	Ile	Tyr
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	Asp	Thr	Ser	Asn	Leu	Ala	Ser	Gly	Val	Pro	Val	Arg	Phe	Ser	Gly	Ser
15		50					55					60				
	Gly	Ser	Gly	Thr	Ser	Tyr	Ser	Leu	Thr	Ile	Ser	Arg	Met	Glu	Ala	Glu
	65					70					75					80
20	Asp	Ala	Ala	Thr	Tyr	Tyr	Cys	Gln	Gln	Trp	Ser	Gly	Tyr	Pro	Tyr	Thr
					85					90					95	
	Phe	Gly	Gly	Gly	Thr	Lys	Leu	Glu	Ile	Lys	Arg	Thr	Val	Ala	Ala	Pro
25				100					105					110		
	Ser	Val	Phe	Ile	Phe	Pro	Pro	Ser	Asp	Glu	Gln	Leu	Lys	Ser	Gly	Thr
			115					120					125			
30																
	Ala	Ser	Val	Val	Cys	Leu	Leu	Asn	Asn	Phe	Tyr	Pro	Arg	Glu	Ala	Lys
		130					135					140				
35	Val	Gln	Trp	Lys	Val	Asp	Asn	Ala	Leu	Gln	Ser	Gly	Asn	Ser	Gln	Glu
	145					150					155					160
	Ser	Val	Thr	Glu	Gln	Asp	Ser	Lys	Asp	Ser	Thr	Tyr	Ser	Leu	Ser	Ser
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	Thr	Leu	Thr	Leu	Ser	Lys	Ala	Asp	Tyr	Glu	Lys	His	Lys	Val	Tyr	Ala
				180					185					190		
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	Cys	Glu	Val	Thr	His	Gln	Gly	Leu	Ser	Ser	Pro	Val	Thr	Lys	Ser	Phe
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Ala Met Ser Trp Phe Arg Gln Ser Pro Glu Lys Arg Leu Glu Trp Val  
35 40 45

Ala Glu Ile Ser Ser Gly Gly Ser Tyr Thr Tyr His Pro His Thr Val  
50 55 60

20

Thr Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr  
65 70 75 80

25

Leu Glu Met Ser Ser Leu Arg Ser Glu Asp Thr Ala Met Tyr Tyr Cys  
85 90 95

30

Ala Arg Gly Leu Trp Gly His Tyr Ala Leu Asp Tyr Trp Gly Gln Gly  
100 105 110

Thr Ser Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe  
115 120 125

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Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu  
130 135 140

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Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp  
145 150 155 160

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu  
165 170 175

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Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser  
180 185 190

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Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro  
195 200 205

55

Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys  
210 215 220

# EP 2 275 443 A1

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					245					250					255		
10																	
	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	His	Glu	Asp	
				260					265					270			
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	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn	
			275					280					285				
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	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ser	Thr	Tyr	Arg	Val	
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25																	
	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu	
	305					310					315					320	
30																	
	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu	Lys	
					325					330					335		
35																	
	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr	
				340					345					350			
40																	
	Leu	Pro	Pro	Ser	Arg	Asp	Glu	Leu	Thr	Lys	Asn	Gln	Val	Ser	Leu	Thr	
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	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	Ile	Ala	Val	Glu	Trp	Glu	
		370					375					380					
50																	
	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	Thr	Thr	Pro	Pro	Val	Leu	
	385					390					395					400	
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	Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	Lys	Leu	Thr	Val	Asp	Lys	
					405					410					415		
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	Ser	Arg	Trp	Gln	Gln	Gly	Asn	Val	Phe	Ser	Cys	Ser	Val	Met	His	Glu	
				420					425					430			
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	Ala	Leu	His	Asn	His	Tyr	Thr	Gln	Lys	Ser	Leu	Ser	Leu	Ser	Pro	Gly	
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 20 25 30  
 Tyr Trp Tyr Gln Gln Lys Pro Gly Ser Ser Pro Arg Leu Leu Ile Tyr  
 35 40 45  
 20  
 Asp Thr Ser Asn His Ala Ser Gly Val Pro Val Arg Phe Ser Gly Ser  
 50 55 60  
 25  
 Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Arg Met Glu Ala Glu  
 65 70 75 80  
 30  
 Asp Ala Ala Thr Tyr Tyr Cys Gln His Trp Ser Gly His Pro Tyr Thr  
 85 90 95  
 Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg Thr Val Ala Ala Pro  
 100 105 110  
 35  
 Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr  
 115 120 125  
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 Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys  
 130 135 140  
 Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu  
 145 150 155 160  
 45  
 Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser  
 165 170 175  
 50  
 Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala  
 180 185 190  
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 Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe  
 195 200 205



# EP 2 275 443 A1

Asn Arg Gly Glu Cys  
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1 5 10 15

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Tyr Trp Tyr Gln Gln Lys Pro Gly Ser Ser Pro Arg Leu Leu Ile Tyr  
35 40 45

25

Asp Thr Ser His His Ala Ser Gly Val Pro Val Arg Phe Ser Gly Ser  
50 55 60

30

Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Arg Met Glu Ala Glu  
65 70 75 80

Asp Ala Ala Thr Tyr Tyr Cys Gln His Trp Ser Gly His Pro Tyr Thr  
85 90 95

35

Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg Thr Val Ala Ala Pro  
100 105 110

40

Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr  
115 120 125

45

Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys  
130 135 140

Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu  
145 150 155 160

50

Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser  
165 170 175

55

Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala  
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# EP 2 275 443 A1

5 Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe  
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Asn Arg Gly Glu Cys  
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25 Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Gly Tyr  
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30 Tyr Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met  
35 40 45

35 Gly Trp Ile Asn Pro Asn Ser Gly Gly Thr Asn Tyr Ala Gln Lys Phe  
50 55 60

40 Gln Gly Arg Val Thr Met Thr Arg Asp Thr Ser Ile Ser Thr Ala Tyr  
65 70 75 80

45 Met Glu Leu Ser Arg Leu Arg Ser Asp Asp Thr Ala Val Tyr Tyr Cys  
85 90 95

50 Ala Arg Glu Asp Val Val Pro Ala Ala Met Ser Phe Tyr Tyr Gly Met  
100 105 110

55 Asp Val Trp Gly Arg Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr  
115 120 125

Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser  
130 135 140

Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu  
145 150 155 160

Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His

EP 2 275 443 A1

				165					170					175		
5	Thr	Phe	Pro	Ala	Val	Leu	Gln	Ser	Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser
				180					185					190		
	Val	Val	Thr	Val	Pro	Ser	Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr	Ile	Cys
10			195				200						205			
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		210					215					220				
15	Pro	Lys	Ser	Cys	Asp	Lys	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro
	225					230					235					240
	Glu	Leu	Leu	Gly	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys
20					245					250					255	
	Asp	Thr	Leu	Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val
				260					265					270		
25	Asp	Val	Ser	His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp
			275					280					285			
	Gly	Val	Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr
30		290					295					300				
	Asn	Ser	Thr	Tyr	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp
35	305					310					315					320
	Trp	Leu	Asn	Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu
					325					330					335	
40	Pro	Ala	Pro	Ile	Glu	Lys	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg
				340					345					350		
	Glu	Pro	Gln	Val	Tyr	Thr	Leu	Pro	Pro	Ser	Arg	Asp	Glu	Leu	Thr	Lys
45			355					360					365			
	Asn	Gln	Val	Ser	Leu	Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp
		370					375					380				
50	Ile	Ala	Val	Glu	Trp	Glu	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys
	385					390					395					400
	Thr	Thr	Pro	Pro	Val	Leu	Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser
55					405					410					415	

# EP 2 275 443 A1

5 Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser  
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 35 40 45  
 35 Asp Asp Thr Asp Arg Pro Ala Gly Ile Pro Glu Arg Phe Ser Gly Ala  
 50 55 60  
 40 Asn Ser Gly Asn Thr Ala Thr Leu Thr Ile Ser Arg Val Glu Ala Gly  
 65 70 75 80  
 Asp Glu Ala Asp Tyr Tyr Cys Gln Val Trp Asp Ser Ser Thr Asp His  
 85 90 95  
 45 Gly Val Phe Gly Gly Gly Thr Lys Leu Thr Val Leu Gly Gln Pro Lys  
 100 105 110  
 50 Ala Asn Pro Thr Val Thr Leu Phe Pro Pro Ser Ser Glu Glu Leu Gln  
 115 120 125  
 Ala Asn Lys Ala Thr Leu Val Cys Leu Ile Ser Asp Phe Tyr Pro Gly  
 130 135 140  
 55 Ala Val Thr Val Ala Trp Lys Ala Asp Gly Ser Pro Val Lys Ala Gly

# EP 2 275 443 A1

	145		150		155		160									
5	Val	Glu	Thr	Thr	Lys	Pro	Ser	Lys	Gln	Ser	Asn	Asn	Lys	Tyr	Ala	Ala
					165					170					175	
10	Ser	Ser	Tyr	Leu	Ser	Leu	Thr	Pro	Glu	Gln	Trp	Lys	Ser	His	Arg	Ser
				180						185				190		
	Tyr	Ser	Cys	Gln	Val	Thr	His	Glu	Gly	Ser	Thr	Val	Glu	Lys	Thr	Val
			195					200					205			
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				20					25					30		
35	His	Met	His	Trp	Val	Arg	Gln	Ala	Pro	Gly	Gln	Gly	Leu	Glu	Trp	Met
			35					40					45			
40	Gly	Trp	Ile	Asn	Pro	Asn	Ser	Gly	Gly	Thr	Asn	Tyr	Ala	Gln	Lys	Phe
		50					55					60				
	Gln	Gly	Arg	Val	Thr	Met	Thr	Arg	Asp	Thr	Ser	Ile	Ser	Thr	Ala	Tyr
	65					70					75					80
45	Met	Glu	Leu	Ser	Arg	Leu	Arg	Ser	Asp	Asp	Thr	Ala	Val	Tyr	Tyr	Cys
					85					90					95	
50	Ala	Arg	Glu	Asp	Val	Val	Pro	Ala	Ala	Met	Ser	Phe	Tyr	Tyr	Gly	Met
				100					105					110		
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			115					120					125			
55																

# EP 2 275 443 A1

	Lys	Gly	Pro	Ser	Val	Phe	Pro	Leu	Ala	Pro	Ser	Ser	Lys	Ser	Thr	Ser	
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5	Gly	Gly	Thr	Ala	Ala	Leu	Gly	Cys	Leu	Val	Lys	Asp	Tyr	Phe	Pro	Glu	
	145					150					155					160	
10	Pro	Val	Thr	Val	Ser	Trp	Asn	Ser	Gly	Ala	Leu	Thr	Ser	Gly	Val	His	
					165					170					175		
15	Thr	Phe	Pro	Ala	Val	Leu	Gln	Ser	Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser	
				180					185					190			
20	Val	Val	Thr	Val	Pro	Ser	Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr	Ile	Cys	
			195				200						205				
25	Asn	Val	Asn	His	Lys	Pro	Ser	Asn	Thr	Lys	Val	Asp	Lys	Lys	Val	Glu	
		210					215					220					
30	Pro	Lys	Ser	Cys	Asp	Lys	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	
	225					230					235					240	
35	Glu	Leu	Leu	Gly	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	
				245						250					255		
40	Asp	Thr	Leu	Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	
				260					265					270			
45	Asp	Val	Ser	His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	
			275					280					285				
50	Gly	Val	Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	
		290					295					300					
55	Asn	Ser	Thr	Tyr	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	
						310					315					320	
60	Trp	Leu	Asn	Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	
					325					330					335		
65	Pro	Ala	Pro	Ile	Glu	Lys	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	
				340					345					350			
70	Glu	Pro	Gln	Val	Tyr	Thr	Leu	Pro	Pro	Ser	Arg	Asp	Glu	Leu	Thr	Lys	
			355					360					365				
75	Asn	Gln	Val	Ser	Leu	Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	

EP 2 275 443 A1

	370	375	380	
5	Ile 385	Ala Val Glu Trp Glu 390	Ser Asn Gly Gln Pro 395	Glu Asn Asn Tyr Lys 400
10	Thr Thr Pro Pro Val 405	Leu Asp Ser Asp Gly 410	Ser Phe Phe Leu Tyr 415	Ser
	Lys Leu Thr Val 420	Asp Lys Ser Arg Trp 425	Gln Gln Gly Asn Val Phe 430	Ser
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35	Thr Leu Ser 20	Leu Thr Cys Ala Val 25	Ser Gly Tyr Ser Ile 30	Ser Asp Asp
40	Gln Ala Trp 35	Ser Trp Val Arg Gln 40	Pro Pro Gly Glu Gly 45	Leu Glu Trp
	Ile 50	Gly Tyr Ile Ser Tyr 55	Ser Gly Ile Thr Asn Tyr 60	Asn Pro Ser Leu
45	Lys 65	Gly Arg Val Thr Ile 70	Ser Arg Asp Thr 75	Lys Asn Gln Phe 80
50	Leu Lys Leu Ser 85	Ser Val Thr Ala Ala 90	Asp Thr Ala Ala Tyr 95	Tyr Cys
55	Ala Arg Ser 100	Leu Ala Arg Thr Thr 105	Ala Met Asp Tyr Trp 110	Gly Glu Gly

EP 2 275 443 A1

	Thr	Leu	Val	Thr	Val	Ser	Ser	Ala	Ser	Thr	Lys	Gly	Pro	Ser	Val	Phe
			115					120					125			
5	Pro	Leu	Ala	Pro	Ser	Ser	Lys	Ser	Thr	Ser	Gly	Gly	Thr	Ala	Ala	Leu
		130					135					140				
10	Gly	Cys	Leu	Val	Lys	Asp	Tyr	Phe	Pro	Glu	Pro	Val	Thr	Val	Ser	Trp
	145					150					155					160
	Asn	Ser	Gly	Ala	Leu	Thr	Ser	Gly	Val	His	Thr	Phe	Pro	Ala	Val	Leu
					165					170					175	
15	Gln	Ser	Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser
				180					185					190		
20	Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr	Ile	Cys	Asn	Val	Asn	His	Lys	Pro
			195					200					205			
	Ser	Asn	Thr	Lys	Val	Asp	Lys	Lys	Val	Glu	Pro	Lys	Ser	Cys	Asp	Lys
25		210					215					220				
	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Pro
	225					230					235					240
30	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met	Ile	Ser
					245					250					255	
	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	His	Glu	Asp
35				260					265					270		
	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn
			275					280					285			
40	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ser	Thr	Tyr	Arg	Val
		290					295					300				
45	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu
	305					310					315					320
	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu	Lys
50					325					330					335	
	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr
				340					345					350		
55	Leu	Pro	Pro	Ser	Arg	Asp	Glu	Leu	Thr	Lys	Asn	Gln	Val	Ser	Leu	Thr



	355		360		365
5	Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu				
	370		375		380
10	Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu				
	385		390		400
15	Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys				
		405		410	415
20	Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu				
		420		425	430
25	Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly				
		435		440	445
30	Lys				

### Claims

1. An antigen-binding molecule having a  $KD(pH5.8)/KD(pH7.4)$  value, defined as the ratio of  $KD$  for the antigen at pH 5.8 and  $KD$  for the antigen at pH 7.4, of 2 or higher.
2. The antigen-binding molecule of claim 1, wherein the  $KD(pH5.8)/KD(pH7.4)$  value is 10 or higher.
3. The antigen-binding molecule of claim 1, wherein the  $KD(pH5.8)/KD(pH7.4)$  value is 40 or higher.
4. The antigen-binding molecule of any one of claims 1 to 3, wherein at least one amino acid of the antigen-binding molecule has been substituted with histidine, or at least one histidine has been inserted into the antigen-binding molecule.
5. The antigen-binding molecule of any one of claims 1 to 4, wherein the antigen-binding molecule has an antagonistic activity.
6. The antigen-binding molecule of any one of claims 1 to 5, wherein the antigen-binding molecule binds to a membrane antigen or a soluble antigen.
7. The antigen-binding molecule of any one of claims 1 to 6, wherein the antigen-binding molecule is an antibody.
8. A pharmaceutical composition comprising the antigen-binding molecule of any one of claims 1 to 7.
9. A method for improving the pharmacokinetics of an antigen-binding molecule by impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4.
10. A method for increasing the number of times of antigen-binding for an antigen-binding molecule by impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4.
11. A method for increasing the number of antigens that can be bound by an antigen-binding molecule by impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4.

12. A method for dissociating within a cell an antigen from an extracellularly-bound antigen-binding molecule by impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4.
13. A method for releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an antigen-free form to the outside of the cell by impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4.
14. A method for increasing the ability of an antigen-binding molecule to eliminate an antigen in plasma by impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4.
15. The method of any one of claims 9 to 14, wherein the  $KD(pH5.8)/KD(pH7.4)$  value, defined as the ratio of  $KD$  for the antigen at pH 5.8 and  $KD$  for the antigen at pH 7.4, is 2 or higher.
16. The method of any one of claims 9 to 14, wherein the  $KD(pH5.8)/KD(pH7.4)$  value is 10 or higher.
17. The method of any one of claims 9 to 14, wherein the  $KD(pH5.8)/KD(pH7.4)$  value is 40 or higher.
18. A method for improving the pharmacokinetics of an antigen-binding molecule by substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule.
19. A method for increasing the number of times of antigen-binding for an antigen-binding molecule by substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule.
20. A method for increasing the number of antigens that can be bound by an antigen-binding molecule by substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule.
21. A method for dissociating within a cell an antigen from an extracellularly-bound antigen-binding molecule by substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule.
22. A method for releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an antigen-free form to the outside of the cell, by substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule.
23. A method for increasing the ability of an antigen-binding molecule to eliminate an antigen in plasma by substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule.
24. The method of any one of claims 18 to 23, wherein the histidine substitution or insertion increases the  $KD(pH5.8)/KD(pH7.4)$  value, defined as the ratio of the antigen-binding activity at pH 5.8 and the antigen-binding activity at pH 7.4, as compared to the  $KD(pH5.8)/KD(pH7.4)$  value before the histidine substitution or insertion.
25. The method of any one of claims 9 to 24, wherein the antigen-binding molecule has an antagonistic activity.
26. The method of any one of claims 9 to 25, wherein the antigen-binding molecule binds to a membrane antigen or a soluble antigen.
27. The method of any one of claims 9 to 26, wherein the antigen-binding molecule is an antibody.
28. A method of screening for an antigen-binding molecule, which comprises the steps of:
  - (a) determining the antigen-binding activity of an antigen-binding molecule at pH 6.7 to pH 10.0;
  - (b) determining the antigen-binding activity of the antigen-binding molecule at pH 4.0 to pH 6.5; and
  - (c) selecting an antigen-binding molecule whose antigen-binding activity at pH 6.7 to pH 10.0 is greater than the antigen-binding activity at pH 4.0 to pH 6.5.

29. The screening method of claim 28, which comprises the step of selecting an antibody whose antigen-binding activity at pH 6.7 to pH 10.0 is twice or higher that of the antigen-binding activity at pH 4.0 to pH 6.5.

30. A method of screening for an antigen-binding molecule, which comprises the steps of:

- (a) binding an antigen-binding molecule to an antigen under a condition of pH 6.7 to pH 10.0;
- (b) placing the antigen-binding molecule that bound to the antigen of (a) under a condition of pH 4.0 to pH 5.5; and
- (c) obtaining an antigen-binding molecule that dissociated under the condition of pH 4.0 to pH 6.5.

31. A method of screening for an antigen-binding molecule whose binding activity at a first pH is greater than that at a second pH, which comprises the steps of:

- (a) binding an antigen-binding molecule to an antigen-immobilized column under the condition of a first pH;
- (b) eluting the antigen-binding molecule that had bound to the column at the first pH from the column under the condition of a second pH; and
- (c) collecting the eluted antigen-binding molecule.

32. A method of screening for an antigen-binding molecule whose binding activity at a first pH is greater than that at a second pH, which comprises the steps of:

- (a) binding an antigen-binding molecule library to an antigen-immobilized column under the condition of a first pH;
- (b) eluting the antigen-binding molecule from the column under the condition of a second pH;
- (c) amplifying the gene encoding the eluted antigen-binding molecule; and
- (d) obtaining the eluted antigen-binding molecule.

33. The screening method of claim 31 or 32, wherein the first pH is 6.7 to 10.0 and the second pH is 4.0 to 6.5.

34. The screening method of any one of claims 28 to 33, wherein at least one or more amino acids of the antigen-binding molecule has been substituted with histidine, or at least one histidine has been inserted into the antigen-binding molecule.

35. The screening method of any one of claims 28 to 33, for obtaining an antigen-binding molecule that is superior in retention in the plasma.

36. The screening method of any one of claims 28 to 33, for obtaining an antigen-binding molecule that is capable of binding to an antigen two or more times.

37. The screening method of any one of claims 28 to 33, for obtaining an antigen-binding molecule that is capable of binding to more antigens as compared to the number of its antigen-binding sites.

38. The screening method of any one of claims 28 to 33, for obtaining an antigen-binding molecule that dissociates an extracellularly-bound antigen within a cell.

39. The screening method of any one of claims 28 to 33, for obtaining an antigen-binding molecule that is bound to an antigen and internalized into a cell, and released to the outside of the cell in an antigen-free form.

40. The screening method of any one of claims 28 to 33, for obtaining an antigen-binding molecule that has increased ability to eliminate an antigen in plasma.

41. The screening method of any one of claims 28 to 40, wherein the antigen-binding molecule is used as a pharmaceutical composition.

42. The screening method of any one of claims 28 to 41, wherein the antigen-binding molecule is an antibody.

43. A method for producing an antigen-binding molecule, which comprises the steps of:

- (a) determining the antigen-binding activity of an antigen-binding molecule at pH 6.7 to pH 10.0;
- (b) determining the antigen-binding activity of the antigen-binding molecule at pH 4.0 to pH 6.5;

- (c) selecting the antigen-binding molecule whose antigen-binding activity at pH 6.7 to pH 10.0 is greater than that at pH 4.0 to pH 6.5;
- (d) obtaining the gene encoding the antigen-binding molecule selected in (c); and
- (e) producing the antigen-binding molecule using the gene obtained in (d).

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**44.** A method for producing an antigen-binding molecule, which comprises the steps of:

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- (a) binding an antigen-binding molecule to an antigen under a condition of pH 6.7 to pH 10.0;
- (b) allowing the antigen-binding molecule bound to the antigen of (a) to stand under a condition of pH 4.0 to pH 6.5;
- (c) collecting the antigen-binding molecule that dissociated under the condition of pH 4.0 to pH 6.5;
- (d) obtaining the gene encoding the antigen-binding molecule obtained in (c); and
- (e) producing the antigen-binding molecule using the gene obtained in (d).

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**45.** A method for producing an antigen-binding molecule whose binding activity at a first pH is greater than that at a second pH, which comprises the steps of:

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- (a) binding an antigen-binding molecule to an antigen-immobilized column under the first pH condition;
- (b) eluting the antigen-binding molecule, which is bound to the column at the first pH, from the column under a second pH condition;
- (c) collecting the eluted antigen-binding molecule;
- (d) obtaining the gene encoding the antigen-binding molecule obtained in (c); and
- (e) producing the antigen-binding molecule using the gene obtained in (d).

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**46.** A method for producing an antigen-binding molecule whose binding activity at a first pH is greater than that at a second pH, which comprises the steps of:

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- (a) binding an antigen-binding molecule library to an antigen-immobilized column under the first pH condition;
- (b) eluting the antigen-binding molecule from the column under the second pH condition;
- (c) amplifying the gene encoding the eluted antigen-binding molecule;
- (d) collecting the eluted antigen-binding molecule;
- (e) obtaining the gene encoding the antigen-binding molecule collected in (d); and
- (f) producing the antigen-binding molecule using the gene obtained in (e).

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**47.** The production method of claim 45 or 46, wherein the first pH is 6.7 to 10.0 and the second pH is 4.0 to 6.5.

**48.** The production method of any one of claims 43 to 47, which further comprises the step of substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule.

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**49.** The production method of any one of claims 43 to 48, wherein the antigen-binding molecule is an antibody.

**50.** A pharmaceutical composition comprising an antigen-binding molecule produced by the production method of any one of claims 43 to 49.

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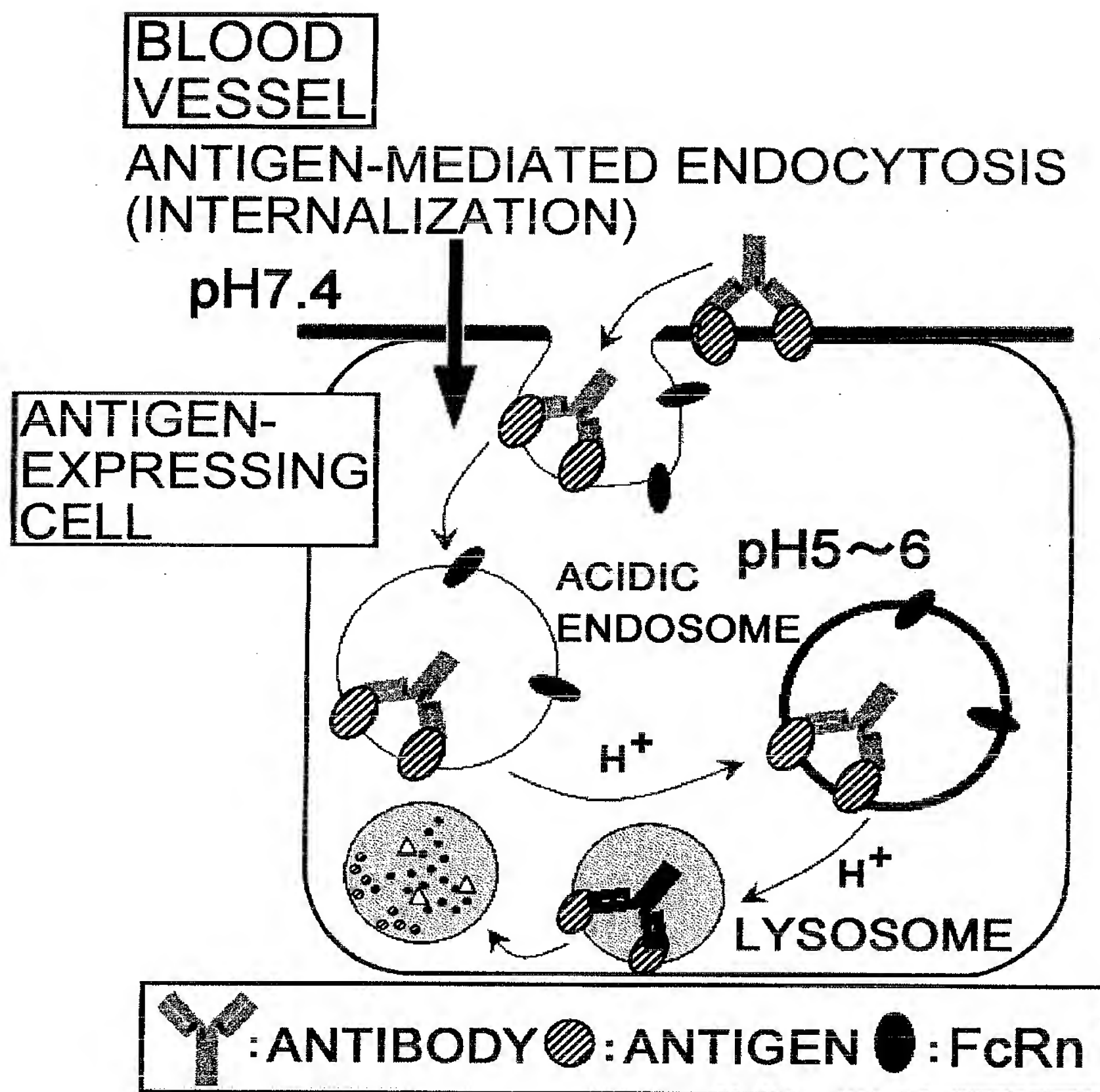


FIG. 1

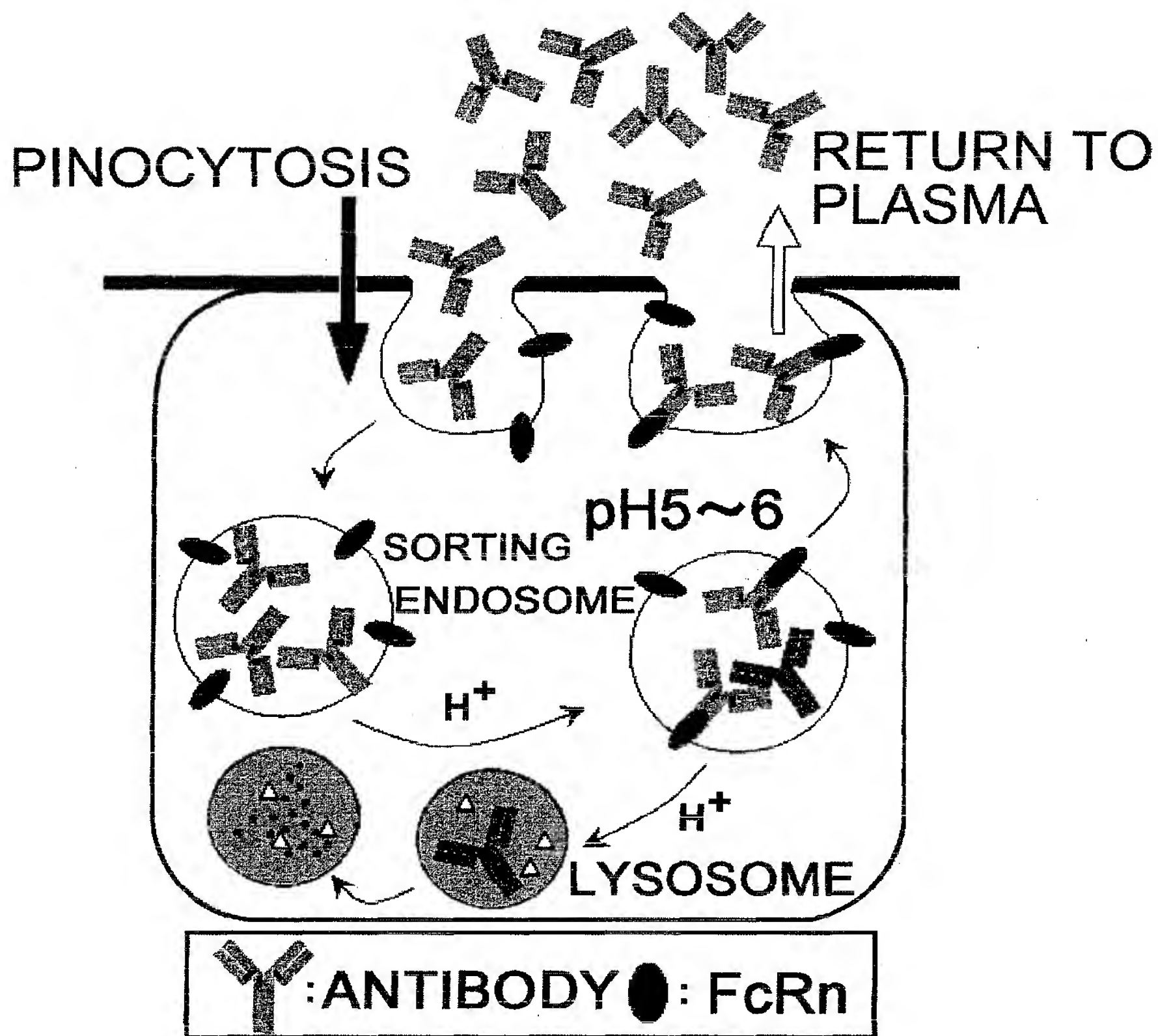


FIG. 2

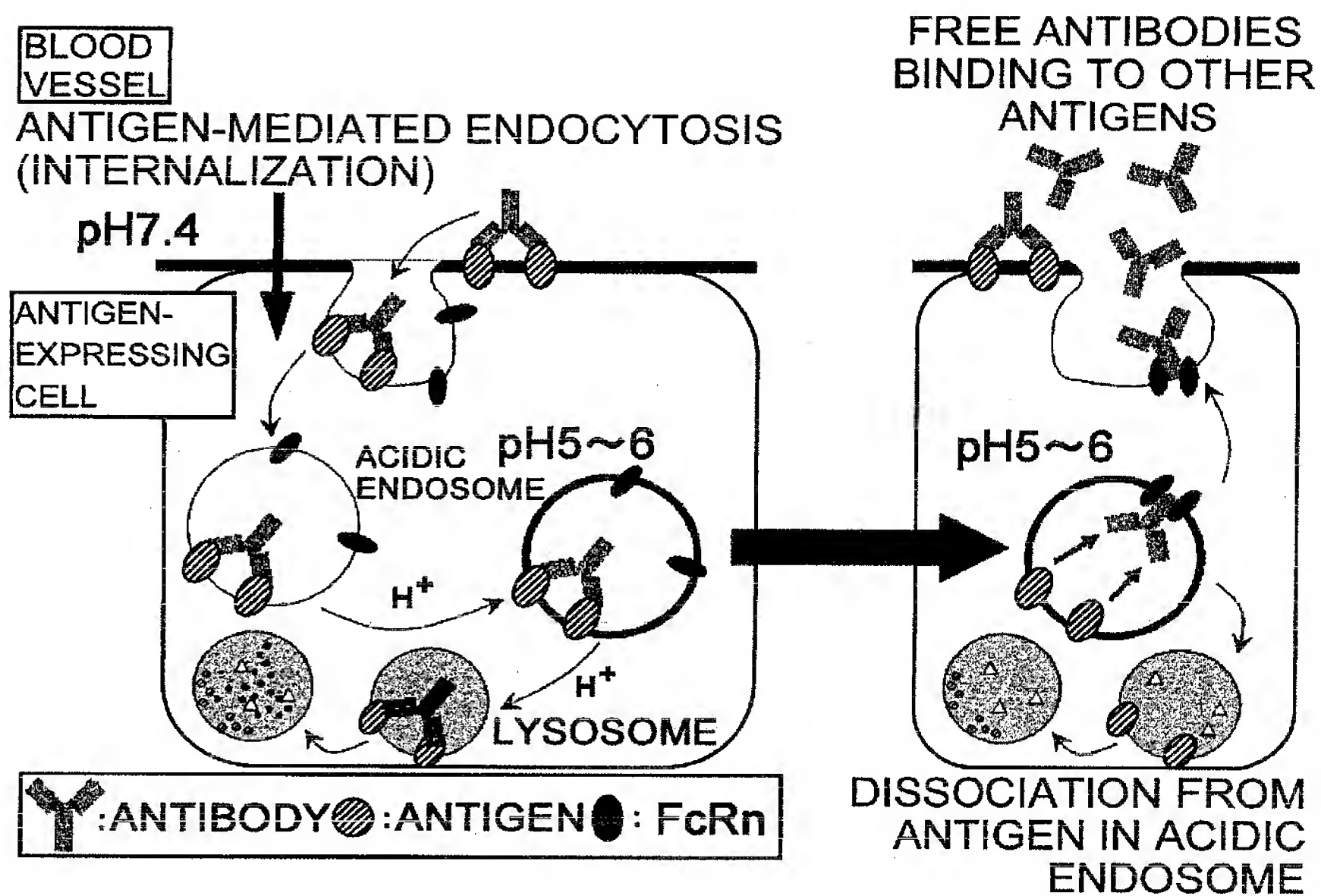


FIG. 3

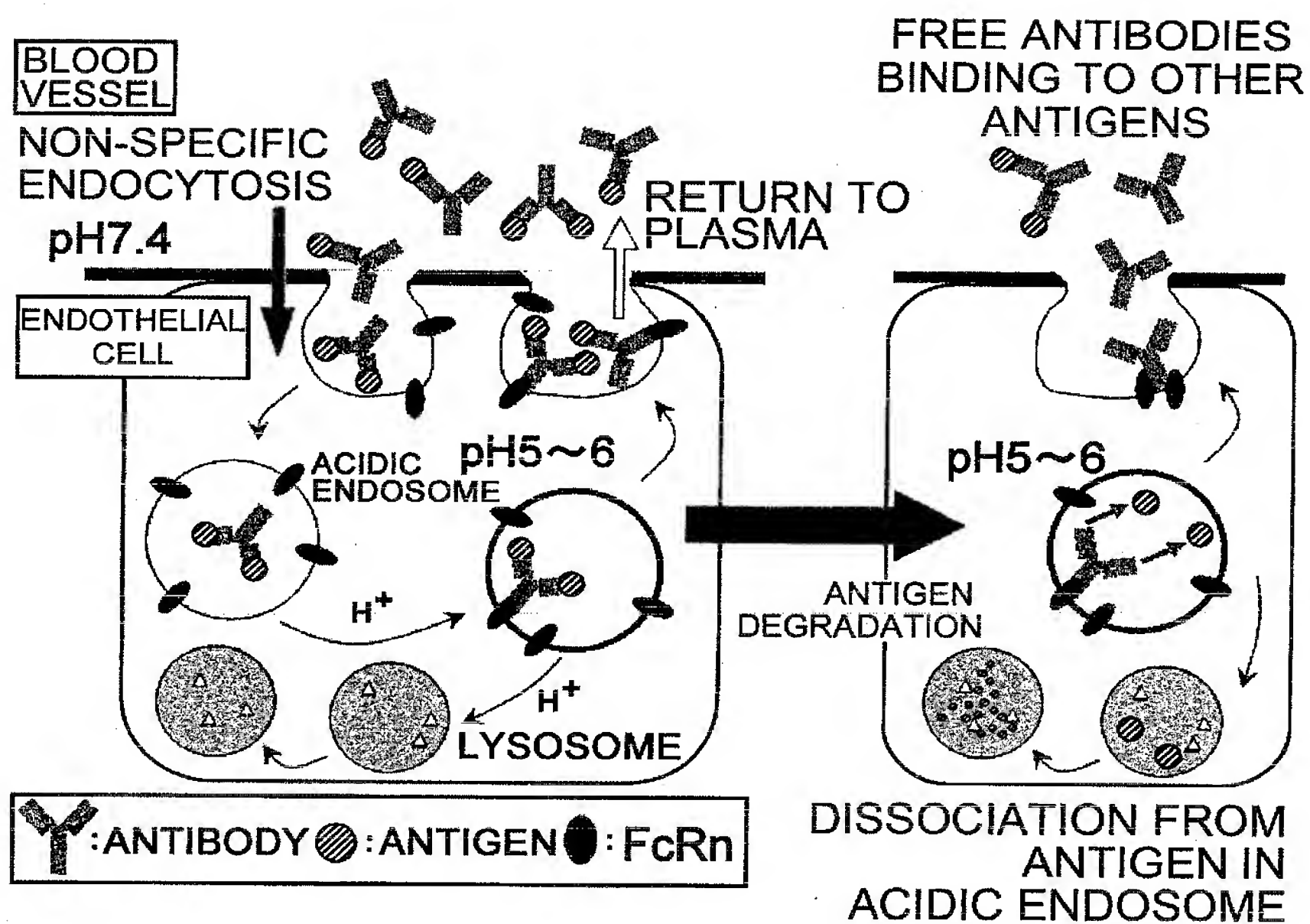


FIG. 4



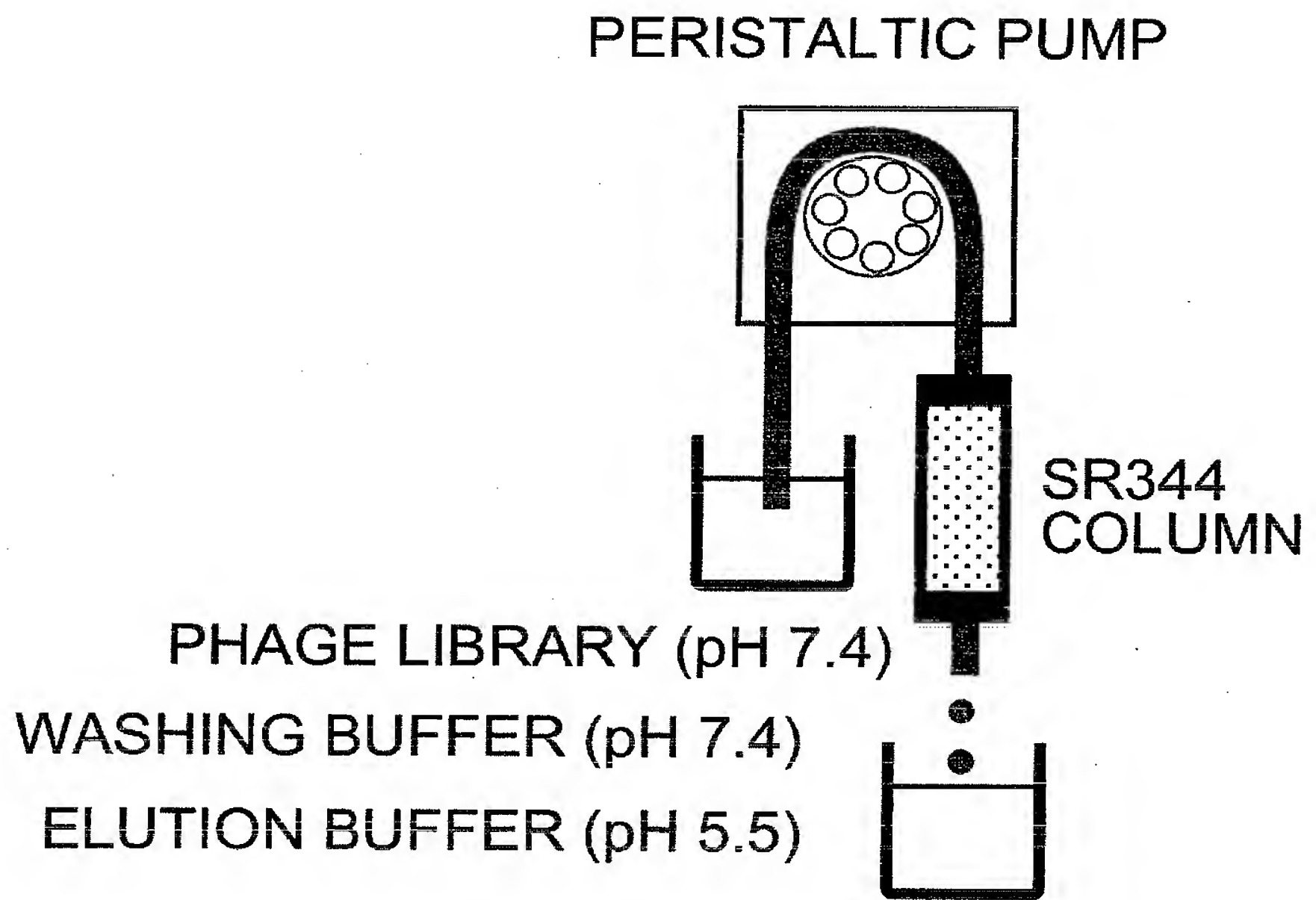


FIG. 5

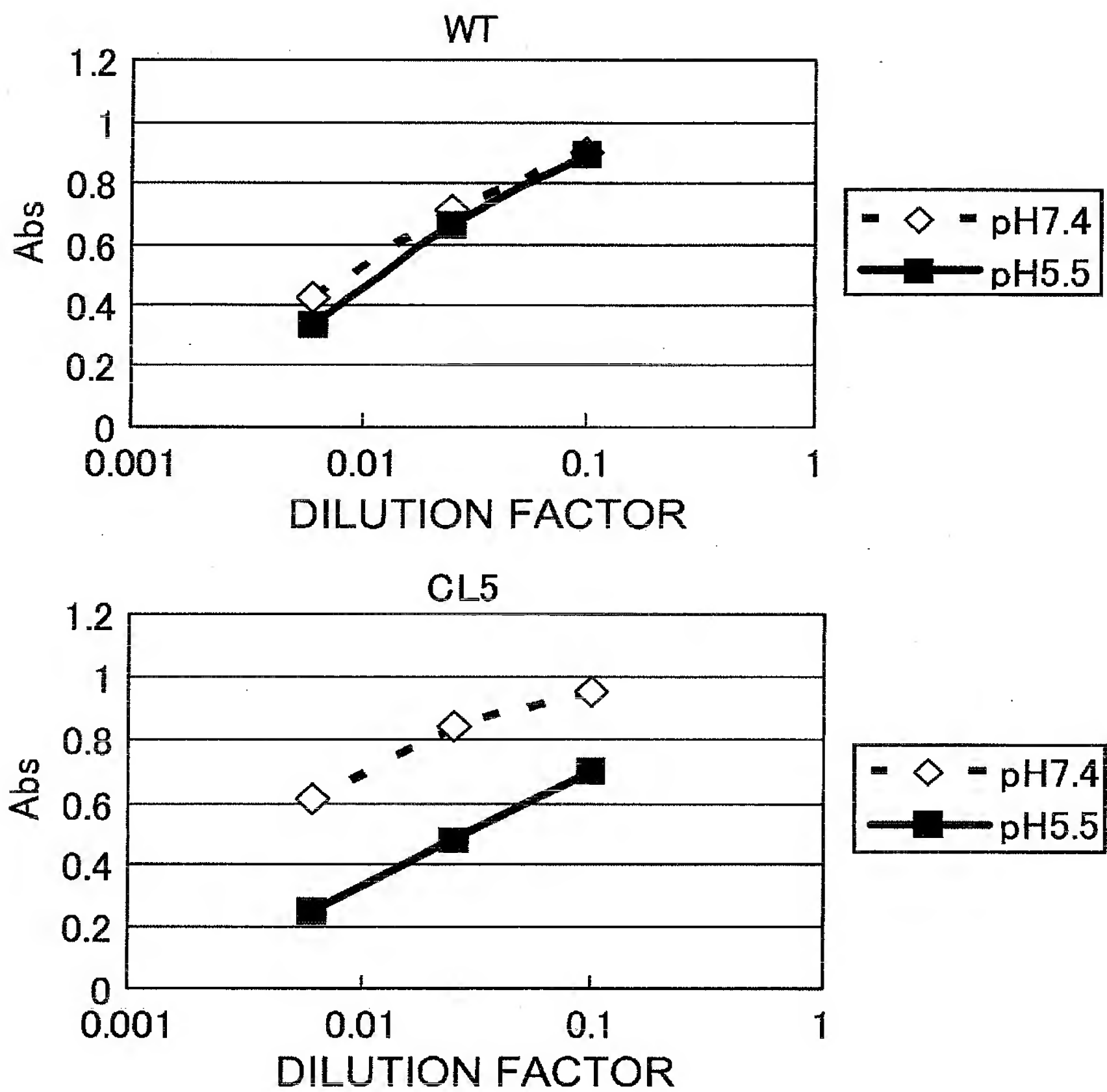


FIG. 6

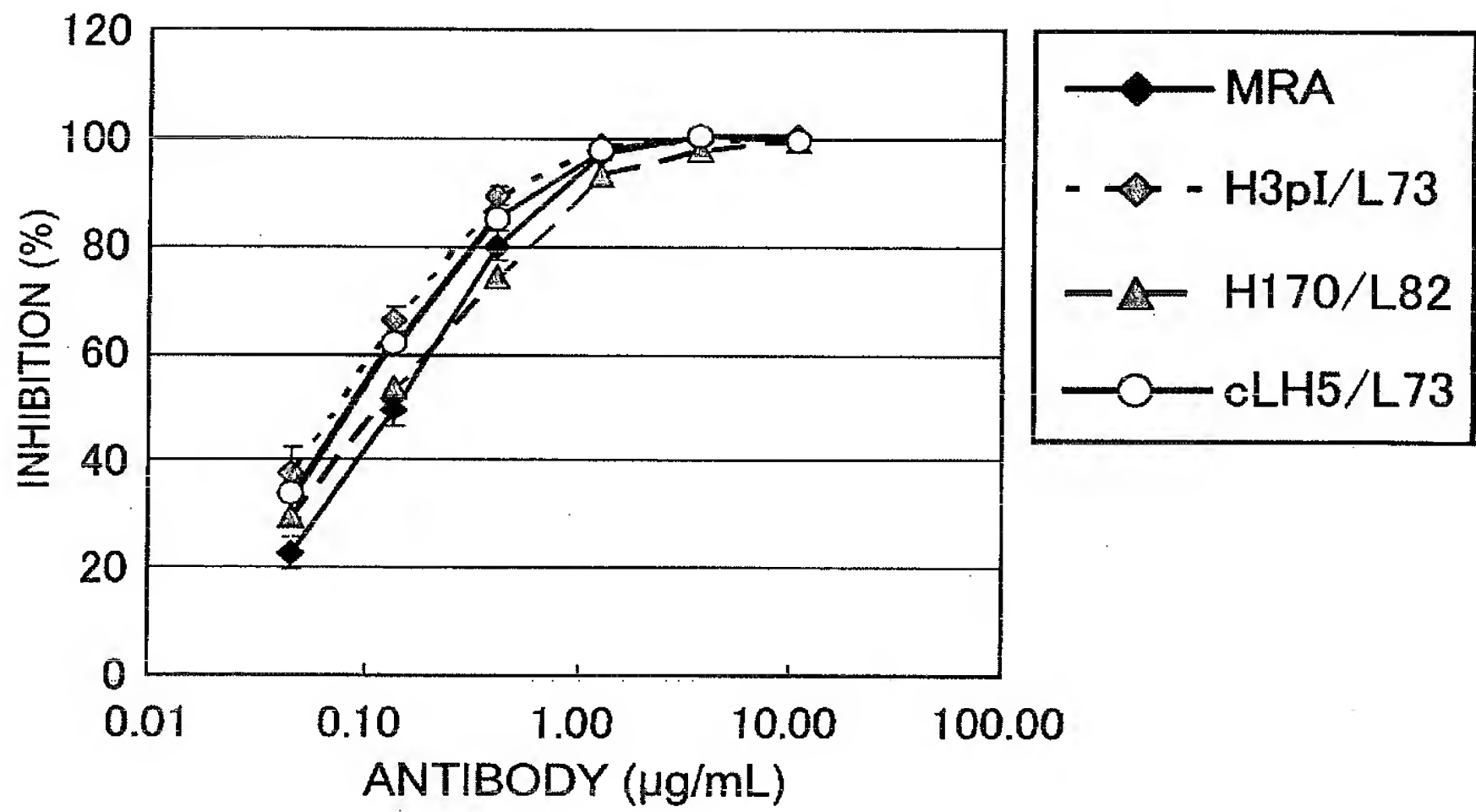


FIG. 7

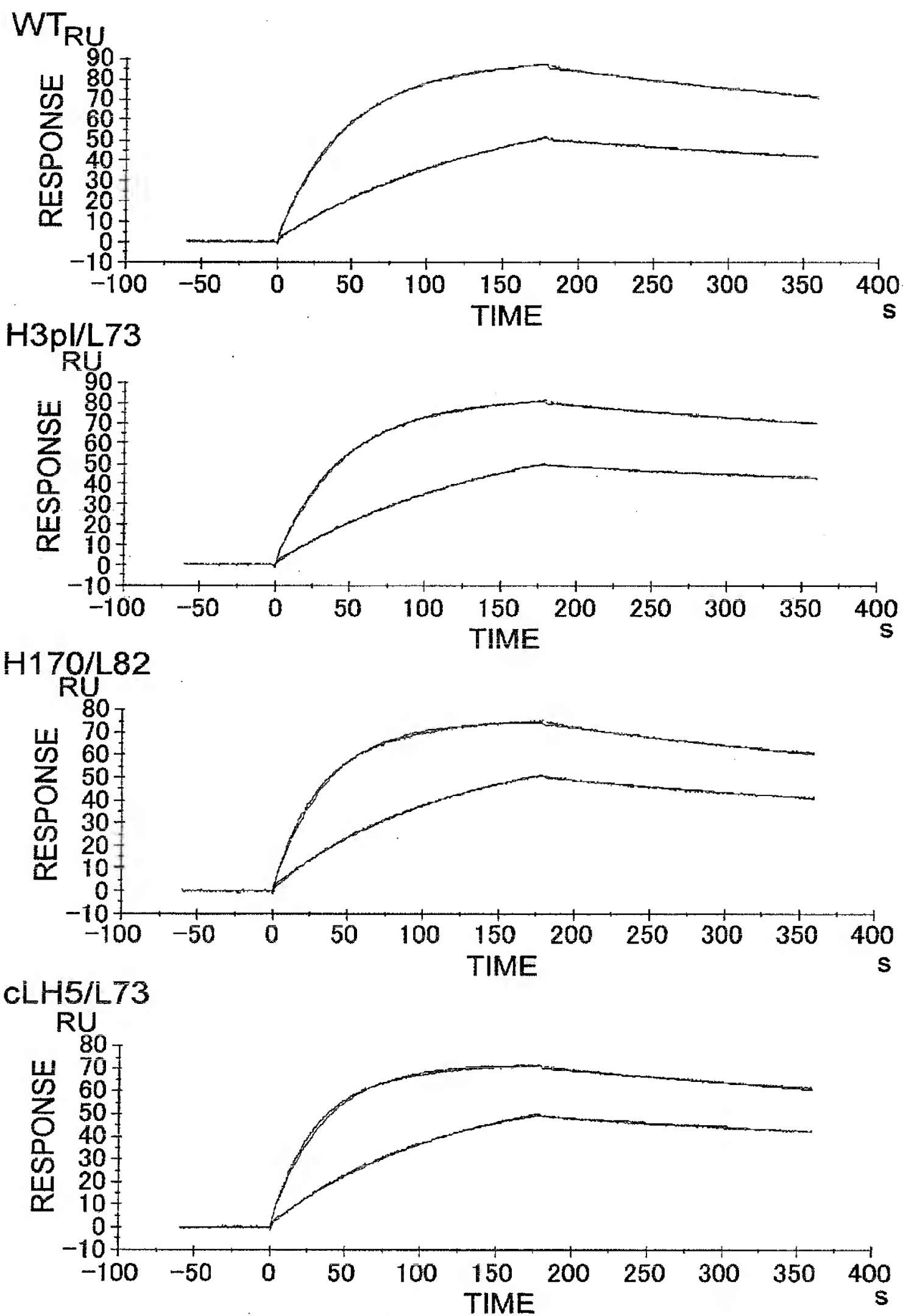


FIG. 8

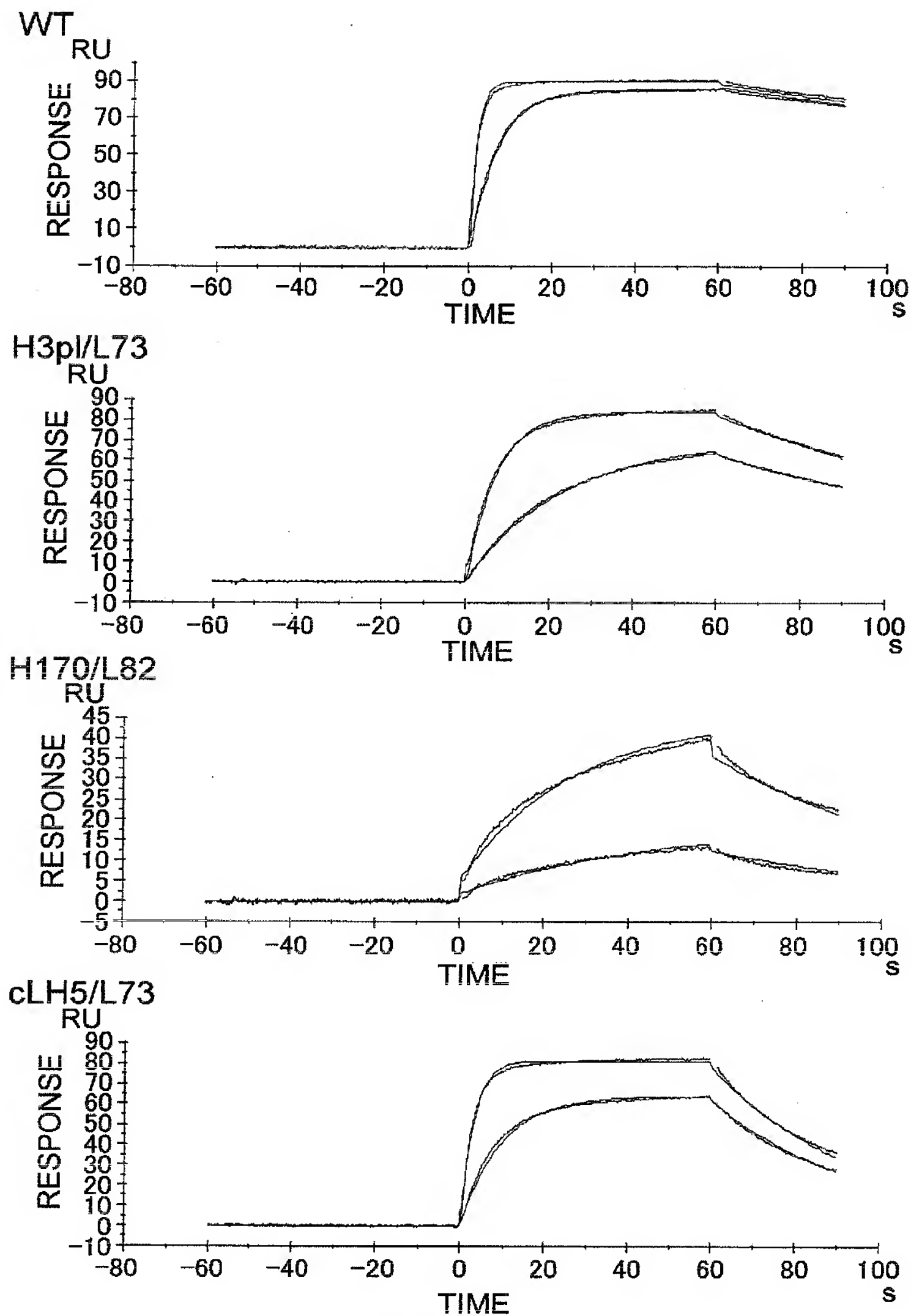


FIG. 9

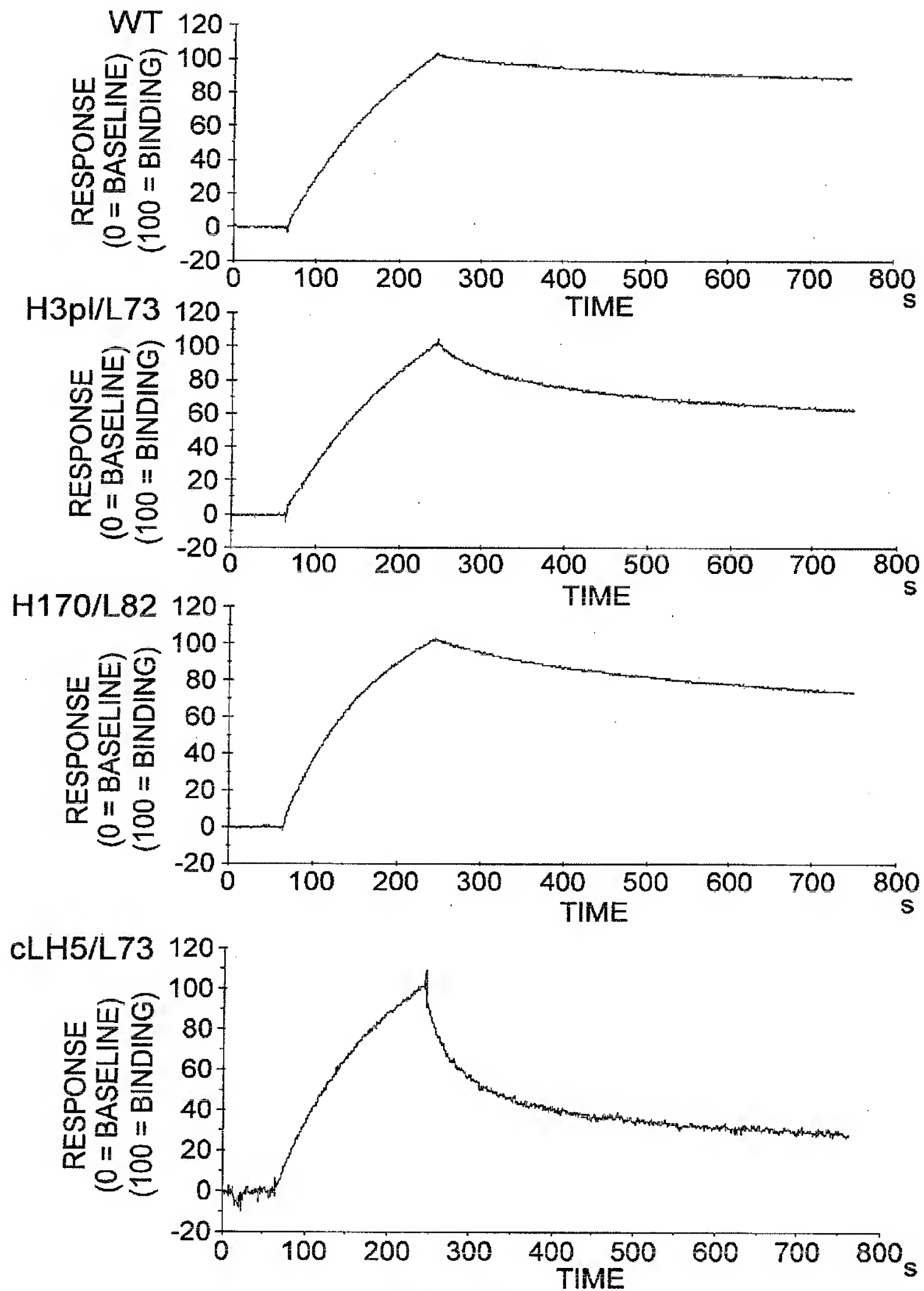


FIG. 10

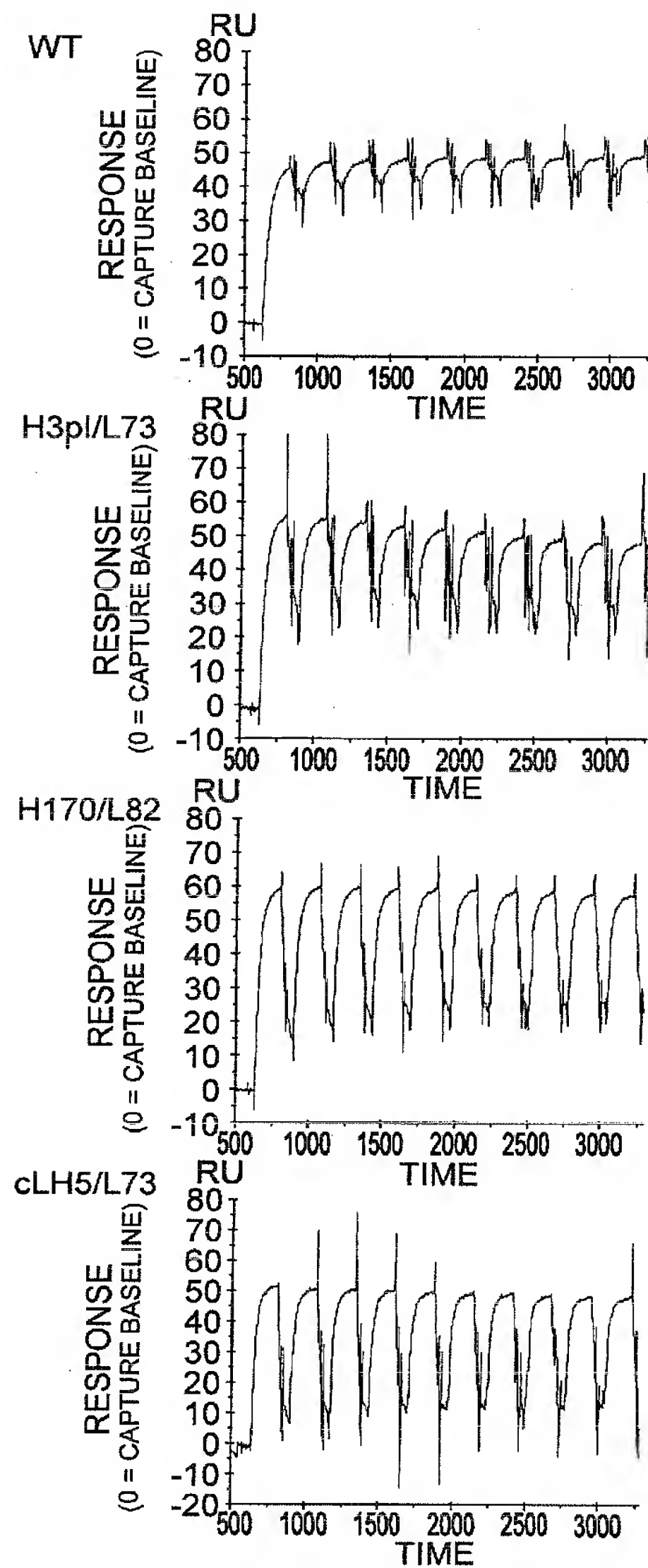


FIG. 11

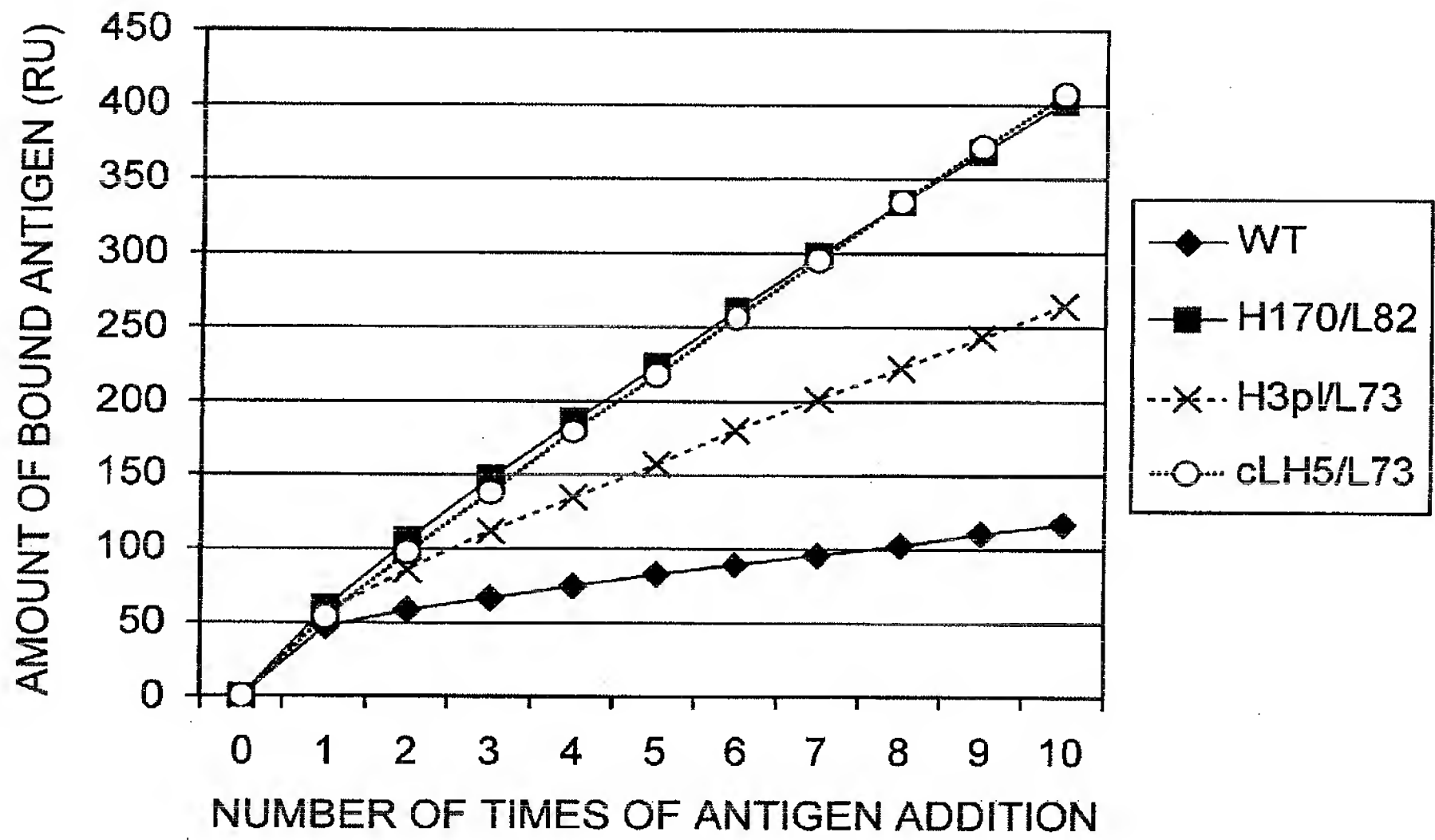


FIG. 12



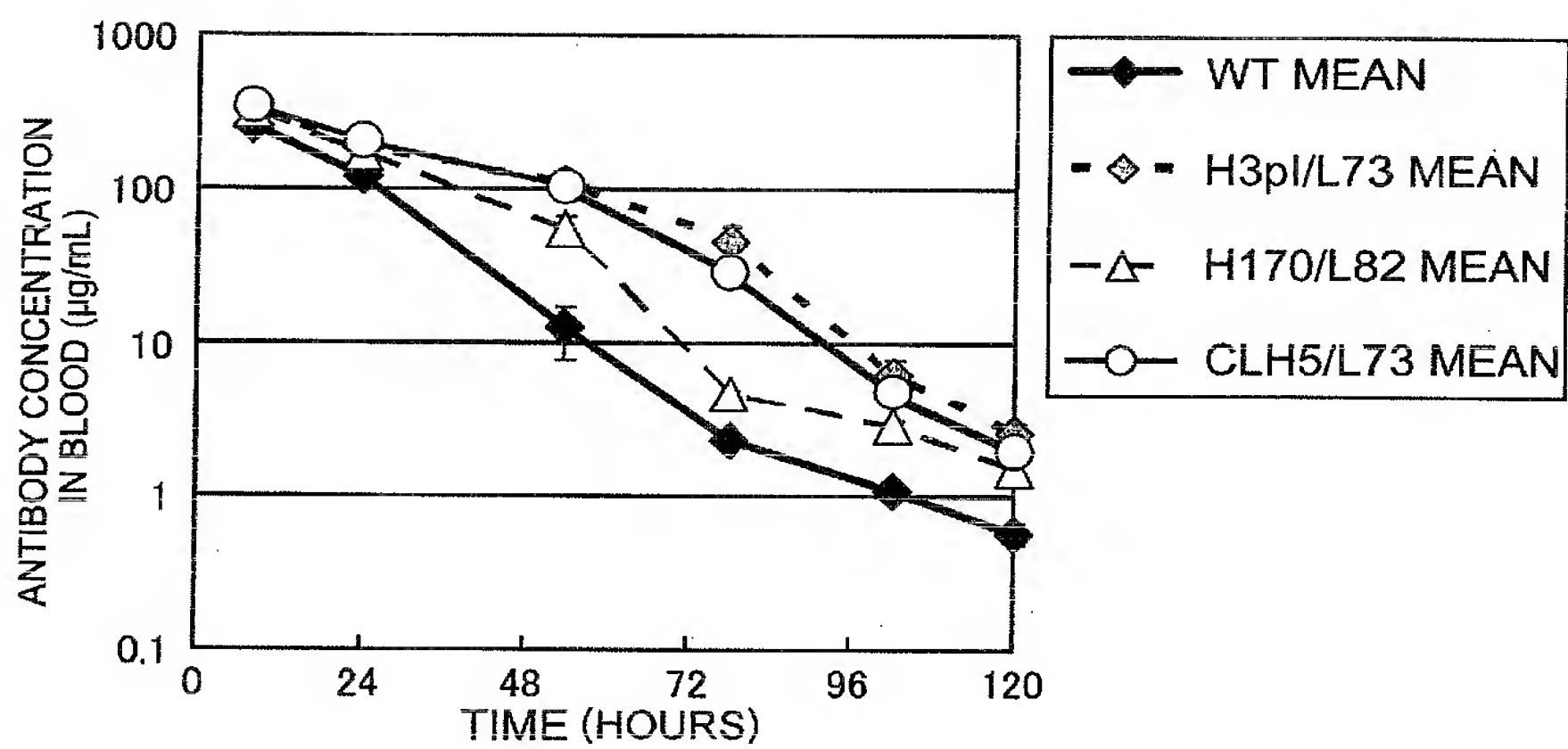


FIG. 13

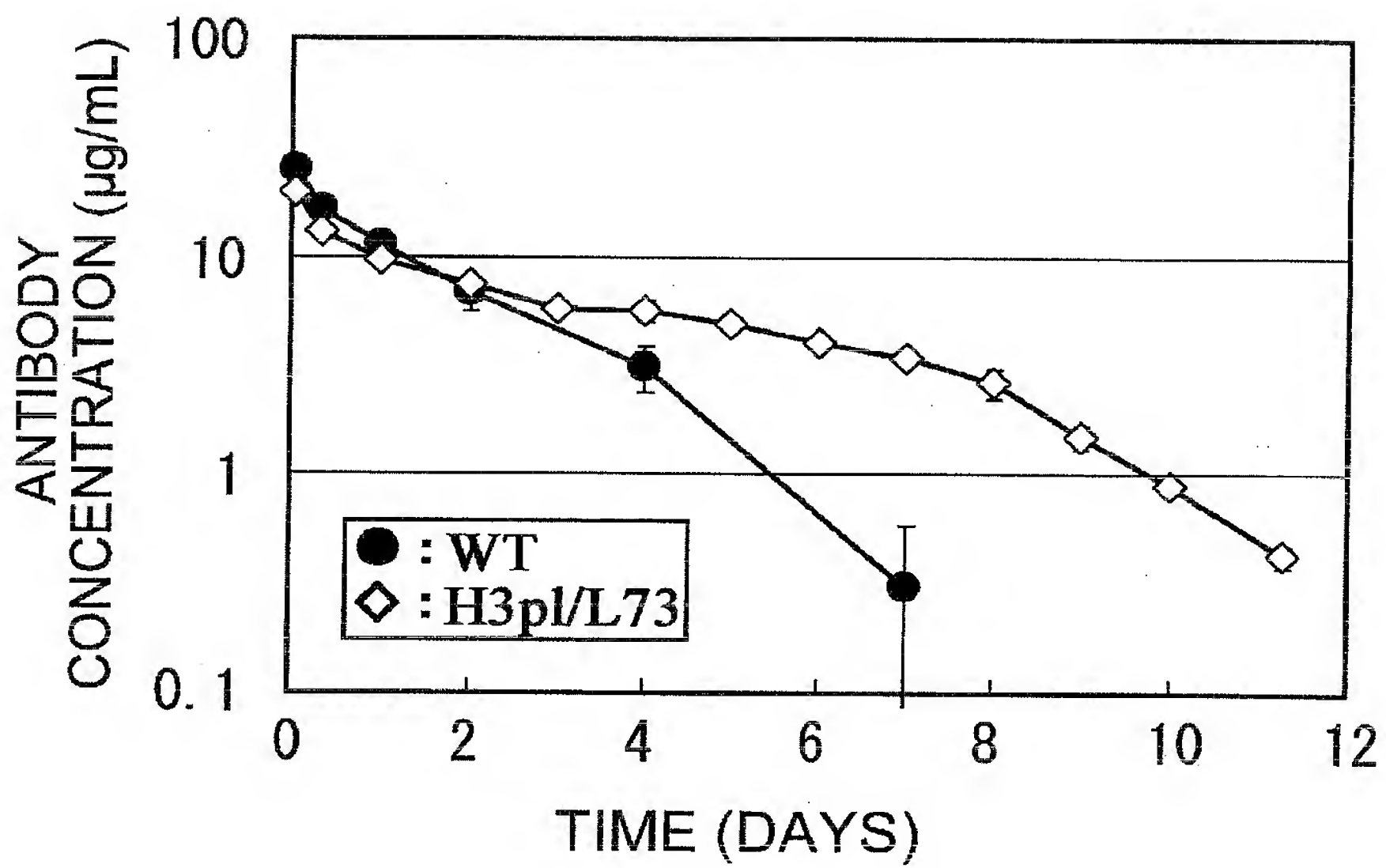


FIG. 14

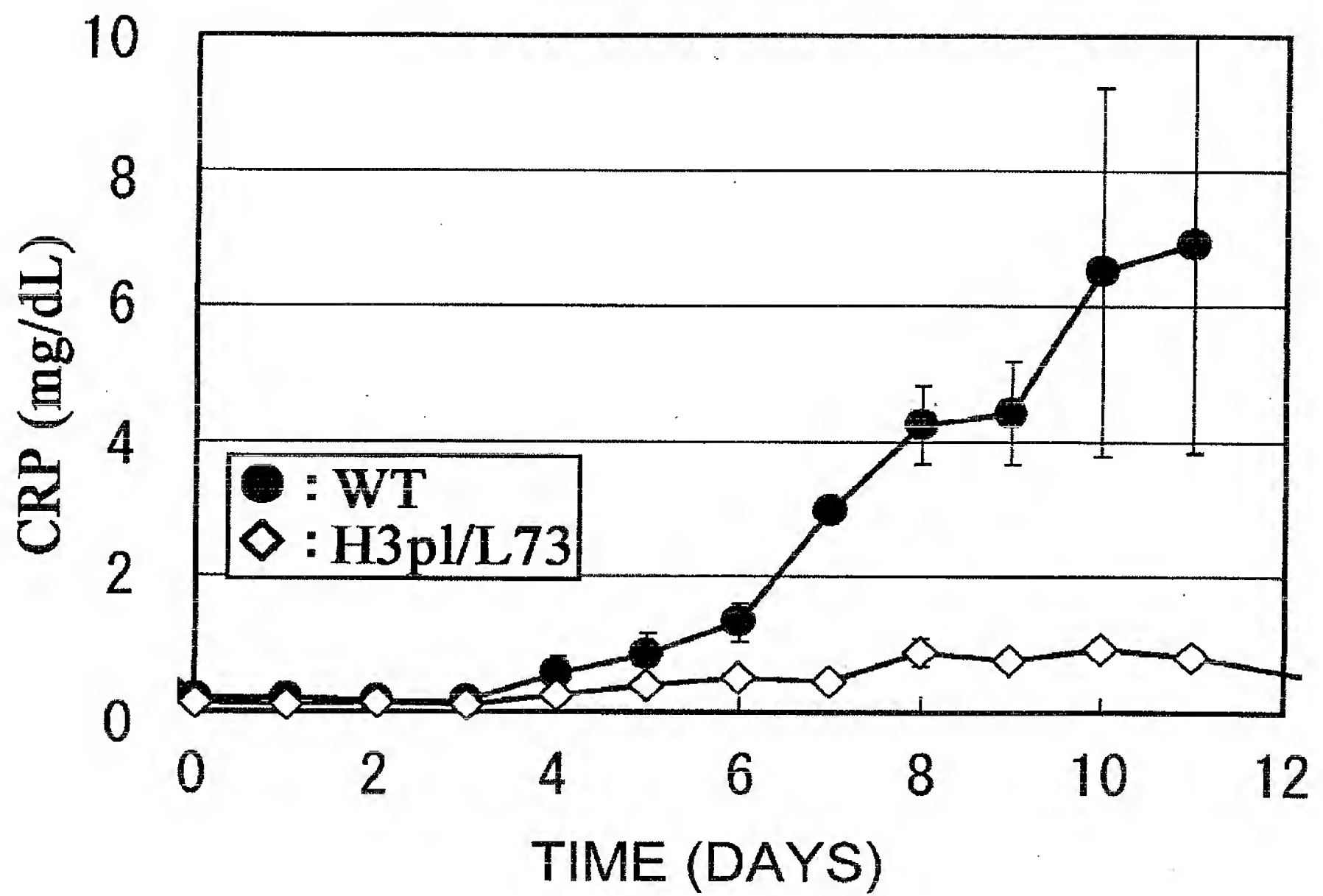


FIG. 15

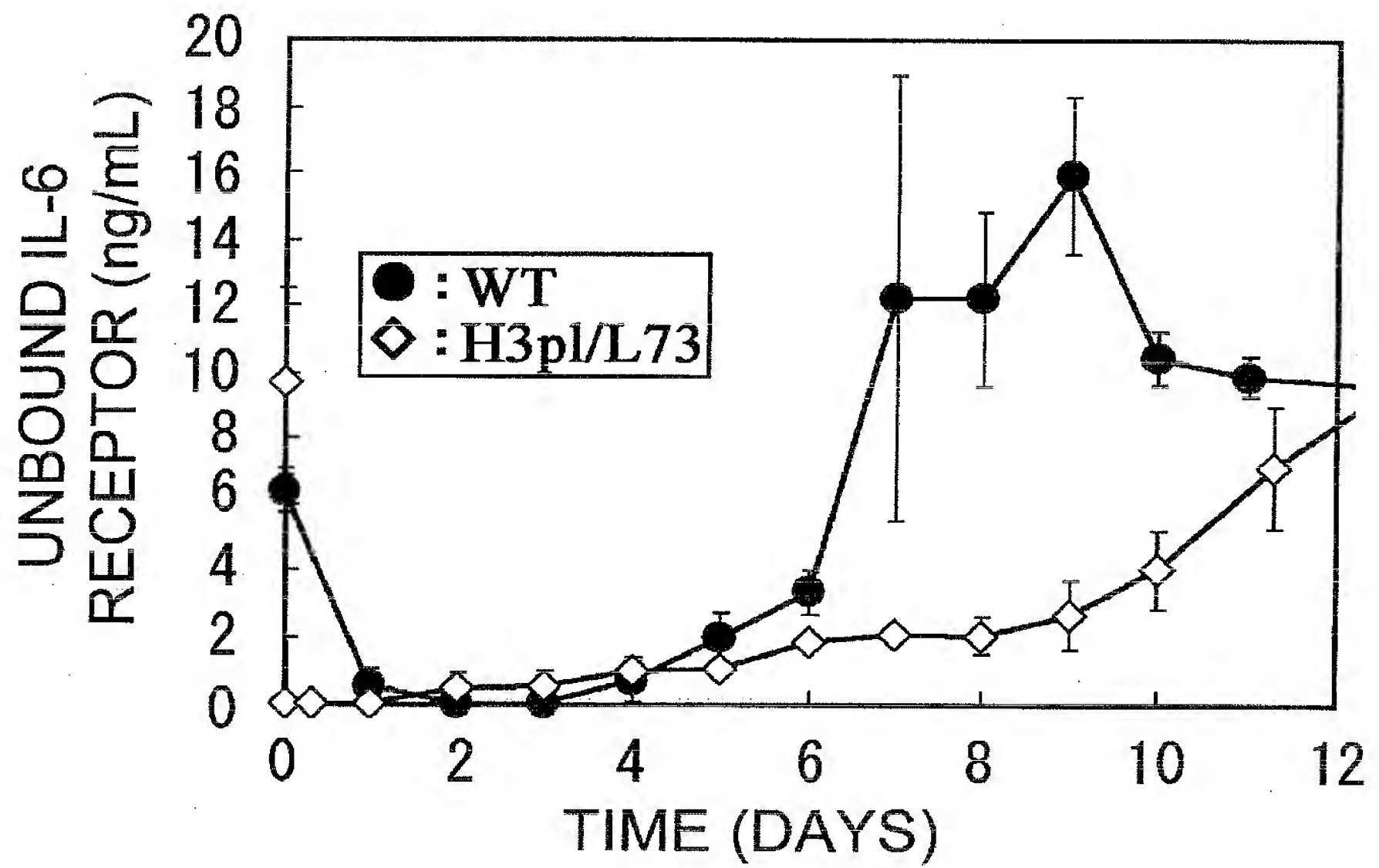


FIG. 16

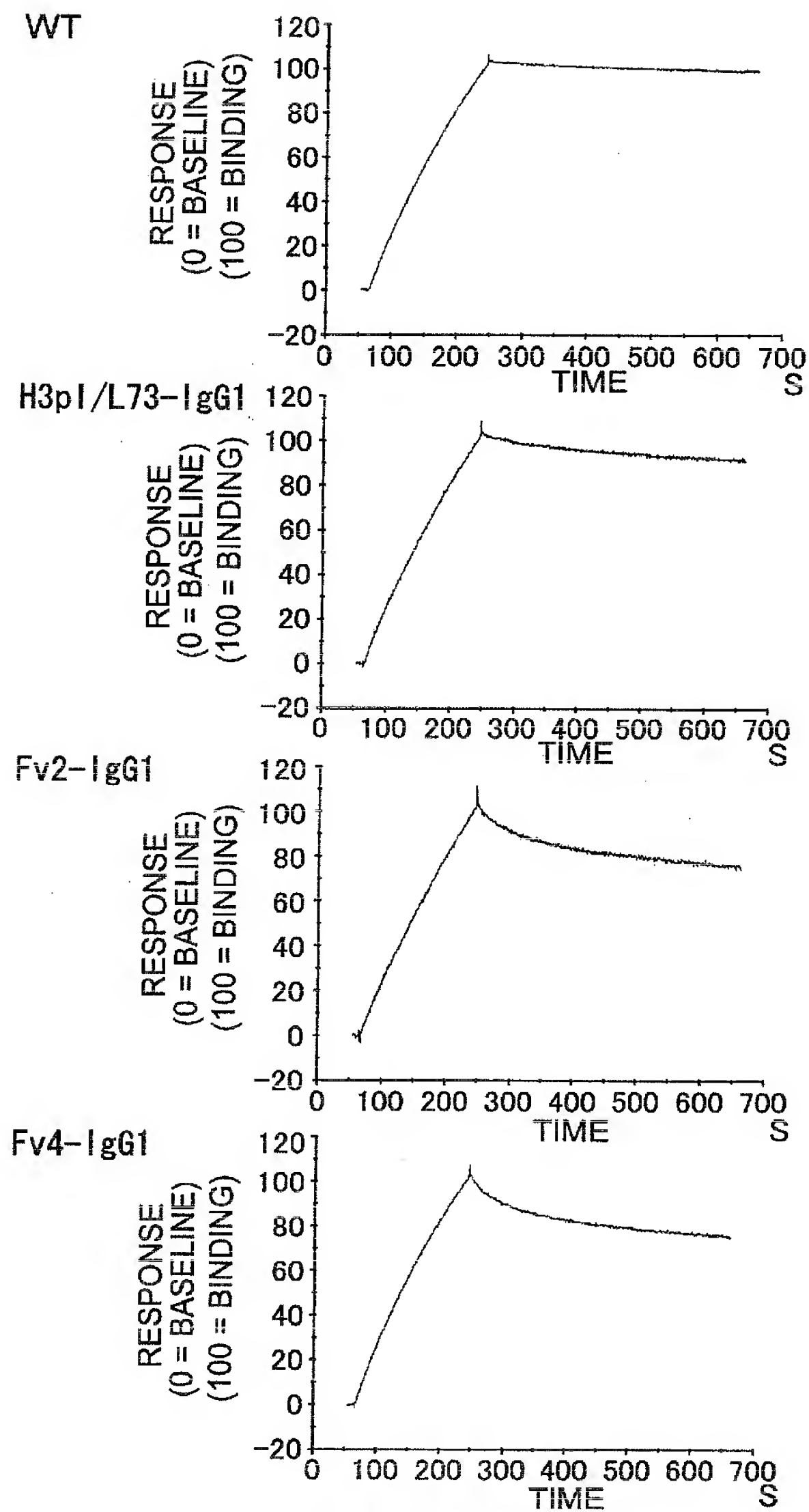


FIG. 17

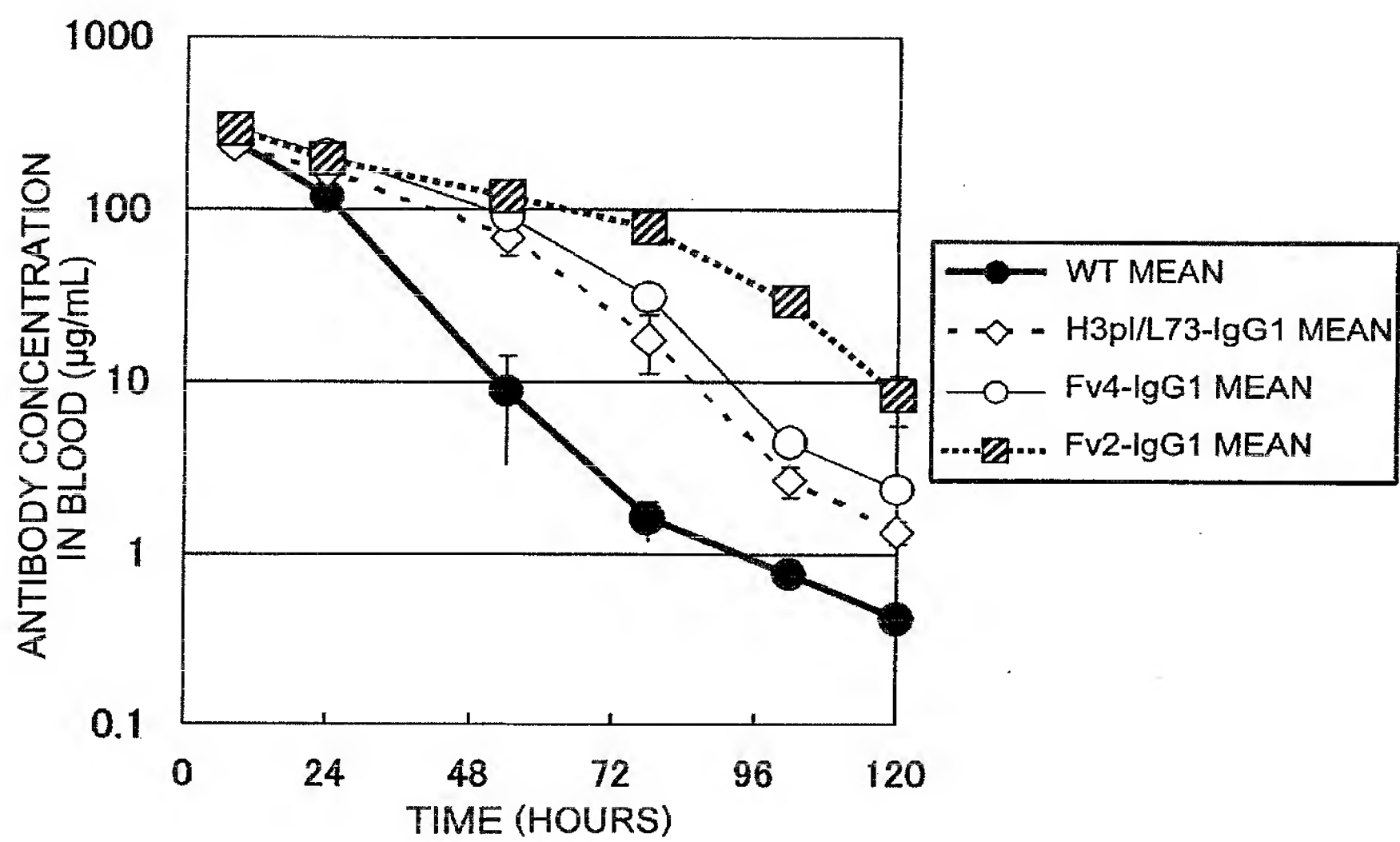


FIG. 18

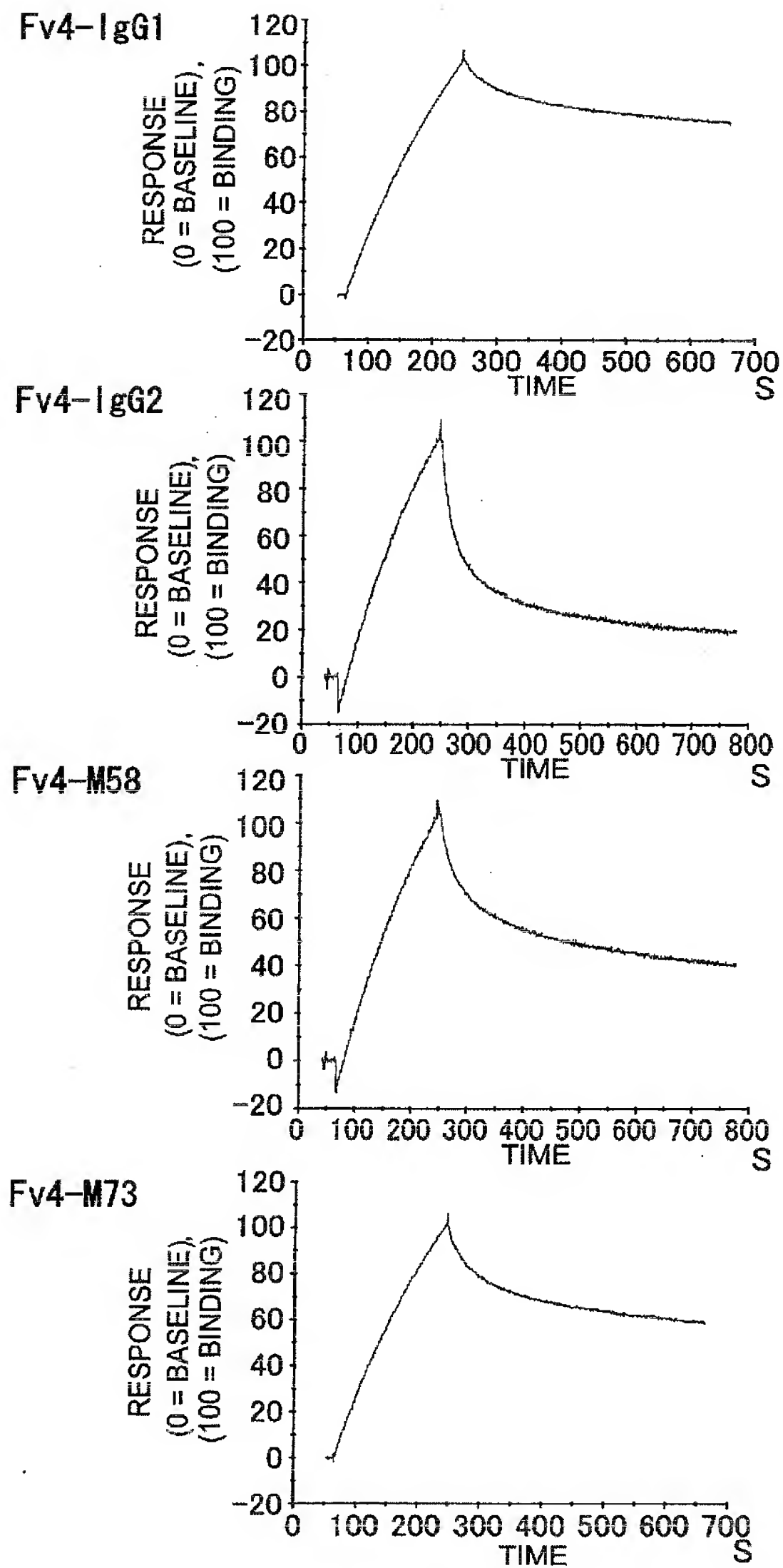


FIG. 19

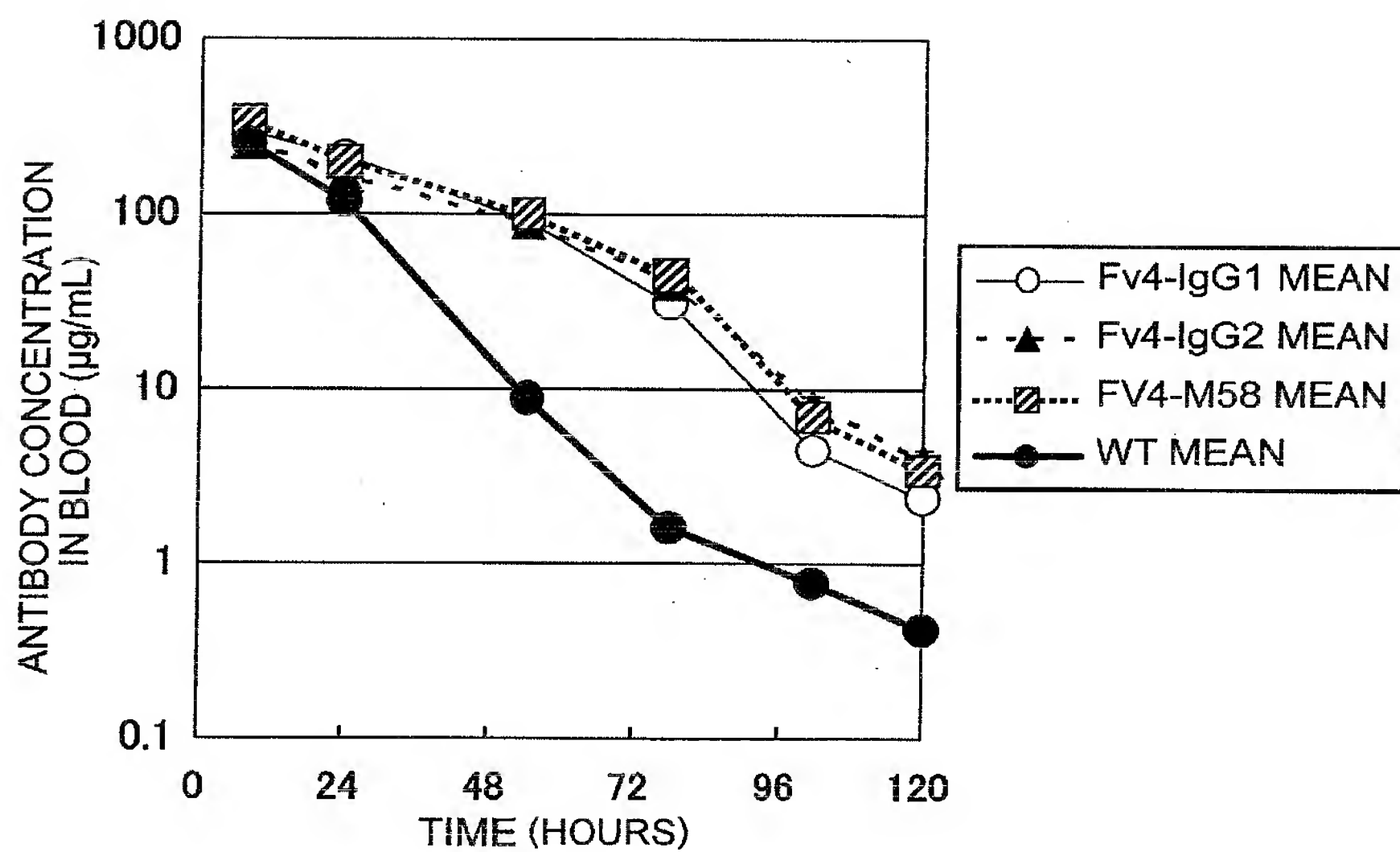


FIG. 20



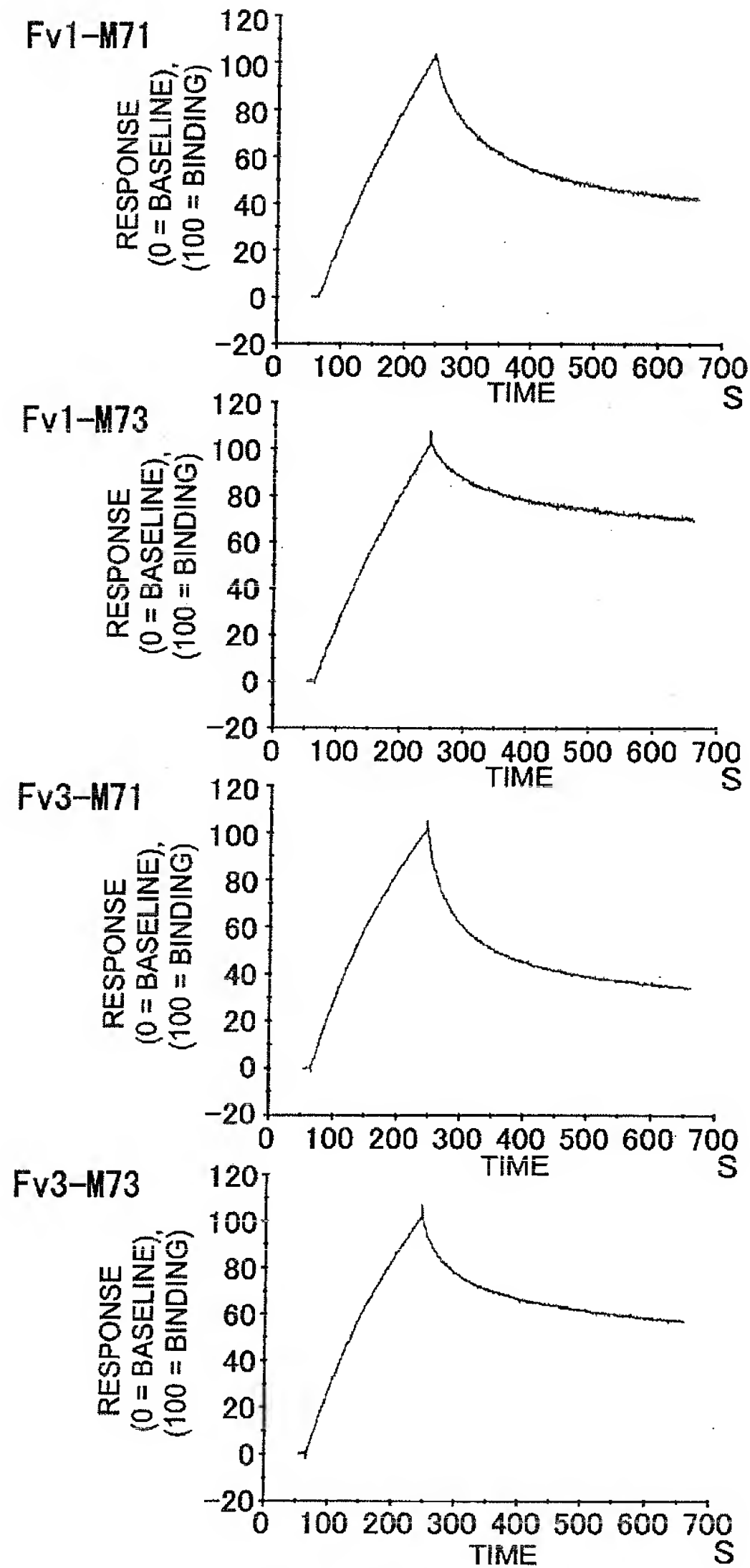


FIG. 21

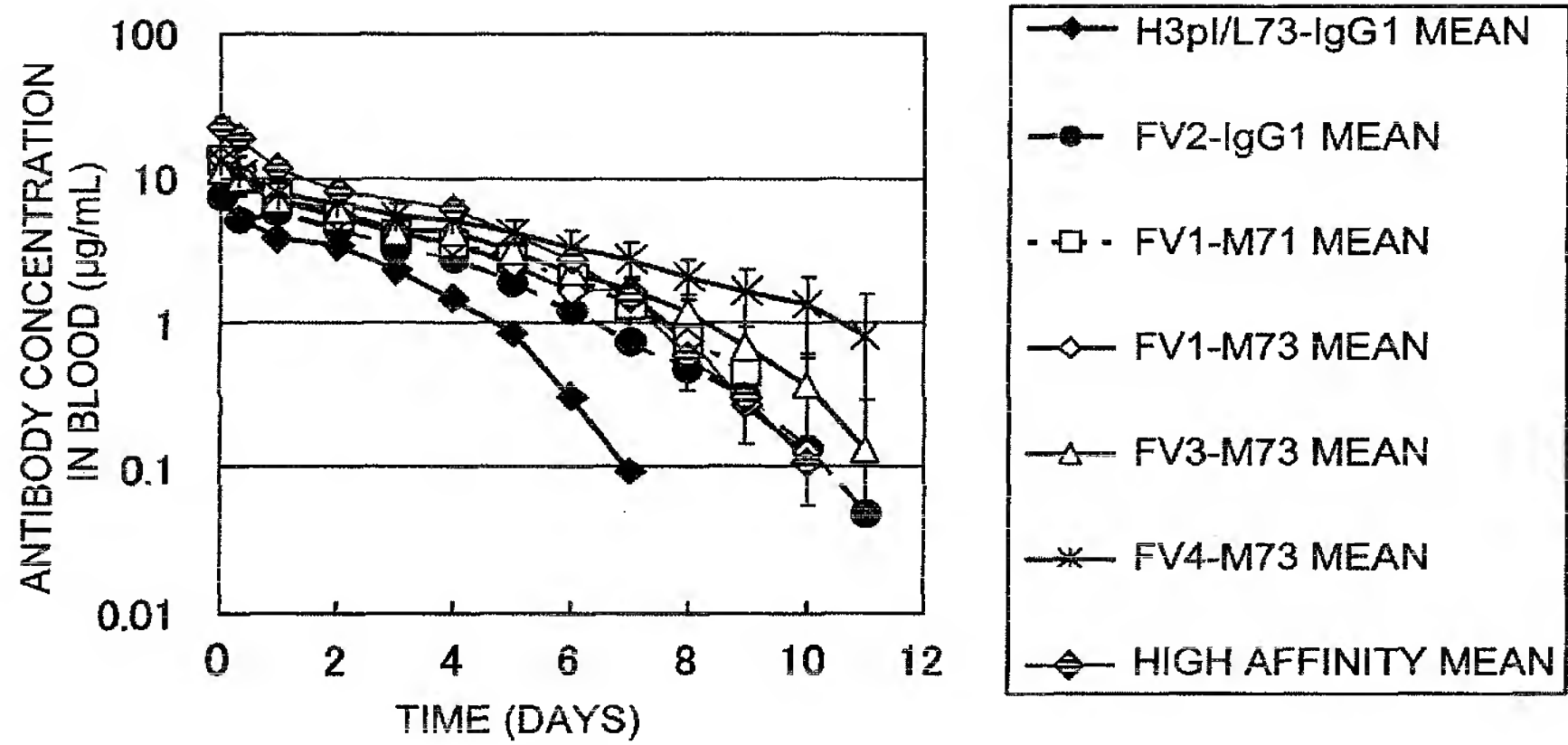


FIG. 22

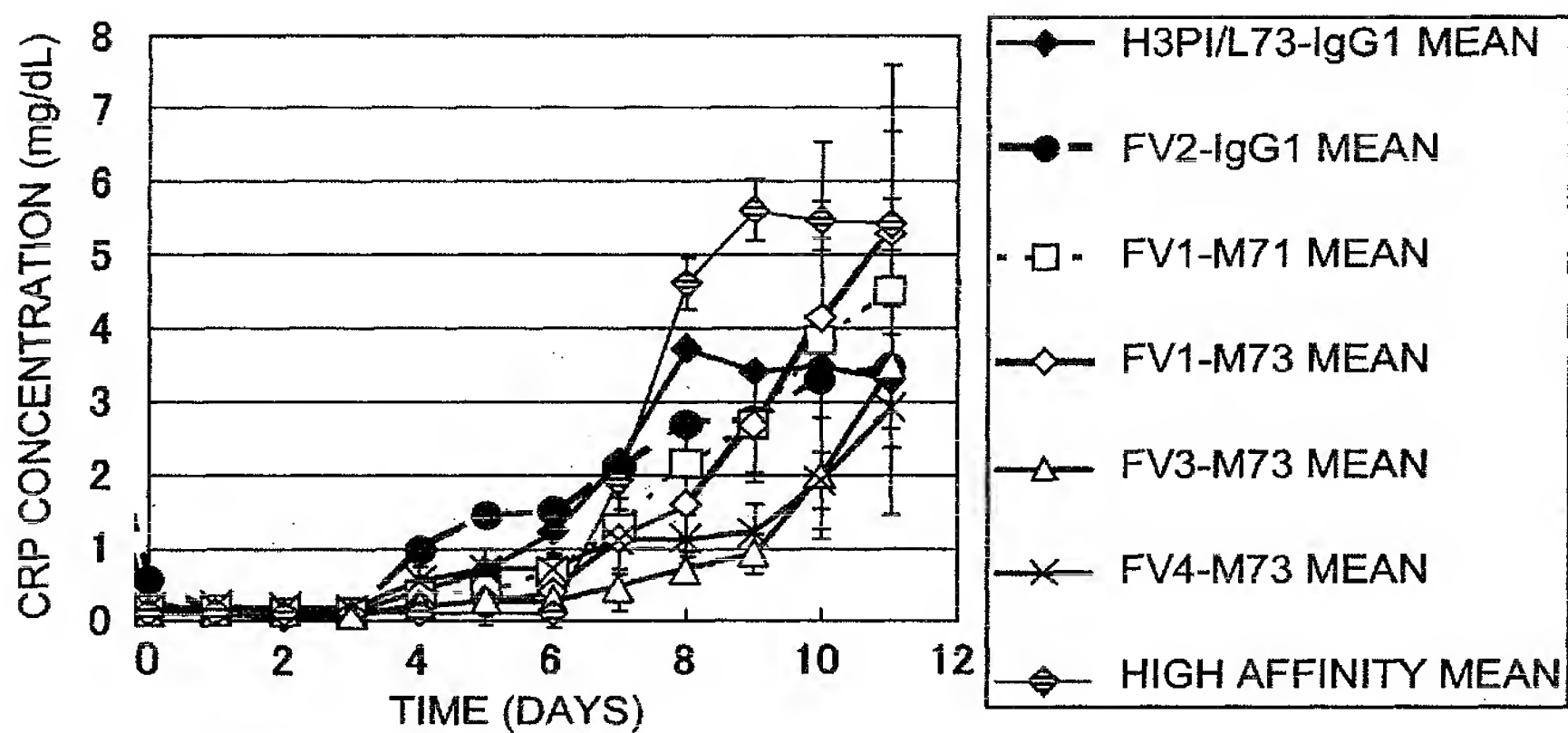


FIG. 23

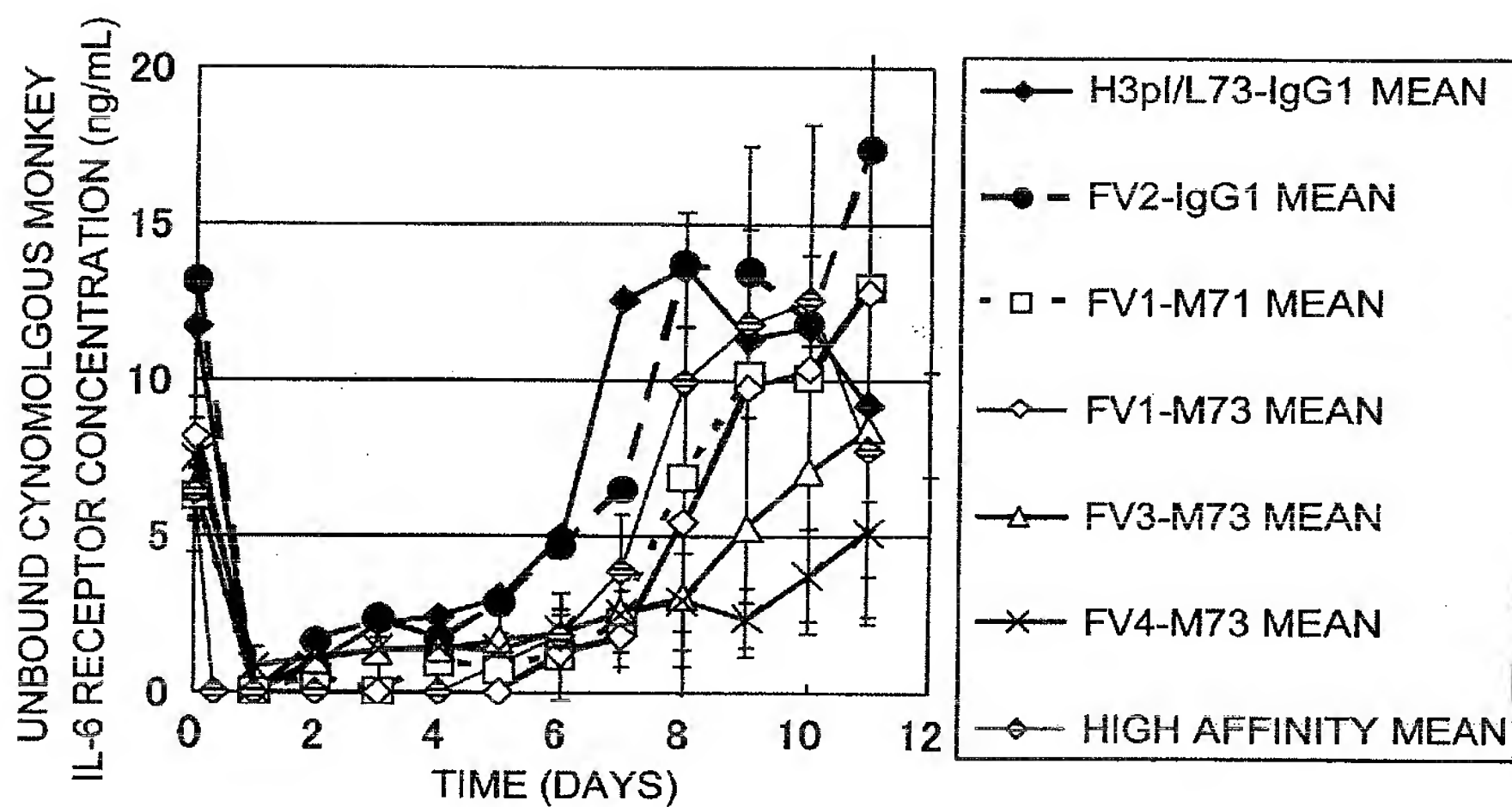


FIG. 24

HEAVY CHAIN									
VH1 : QVQLQESGPGLVKPSSETLSLTCAVSGYSIS	HDHAW	WVRQPPGEGLEWIG	HISYSGITNYPHQLQD						
VH2 : QVQLQESGPGLVKPSSETLSLTCAVSGYSIS	HDHAW	WVRQPPGEGLEWIG	HISYSGITNYPHQLQD						
VH3 : QVQLQESGPGLVKPSSETLSLTCAVSGHSIS	HDHAW	WVRQPPGEGLEWIG	FISYSGITNYPNPSLQG						
VH4 : QVQLQESGPGLVKPSSETLSLTCAVSGHSIS	HDHAW	WVRQPPGEGLEWIG	FISYSGITNYPNPTLQG	*			*	*	*
	FR1	CDR1	FR2	CDR2					
VH1 : RVTISRDTSKNQFSLKLSVTAADTAAYYCAR	FLARITAH	WGEGLVTVSS							
VH2 : RVTISRDTSKNQFSLKLSVTAADTAAYYCAR	FLARITAH	WGEGLVTVSS							
VH3 : RVTISRDNKNTLYLQNNSLRAEDTAVYYCAR	SLARITANDY	WGEGLVTVSS							
VH4 : RVTISRDNKNTLYLQNNSLRAEDTAVYYCAR	SLARITANDY	WGEGLVTVSS							
	*	***	***	*	*	*	*	*	*
	FR3	CDR3	FR4						
LIGHT CHAIN									
VL1 DIQMTQSPSSLSASVGDSTITC	QASRDISSHLN	WYQOKPGKAPPELLIY	YGSHELLS	GVPSRFSGSGSGTDFTFTISSLEAEDAATYYC	GQGNRLPYT	FGQGTKVEIE			
VL2 DIQMTQSPSSLSASVGDSTITC	QASRDISSHLN	WYQOKPGKAPPELLIY	YGSHELLS	GVPSRFSGSGSGTDFTFTISSLEAEDAATYYC	GQGNRLPYT	FGQGTKVEIE			
VL3 DIQMTQSPSSLSASVGDSTITC	QASTDISSHLN	WYQOKPGKAPPELLIY	YGSHELLS	GVPSRFSGSGSGTDFTFTISSLEAEDAATYYC	GQGNRLPYT	FGQGTKVEIE			
	*		*						
FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4			

FIG. 25

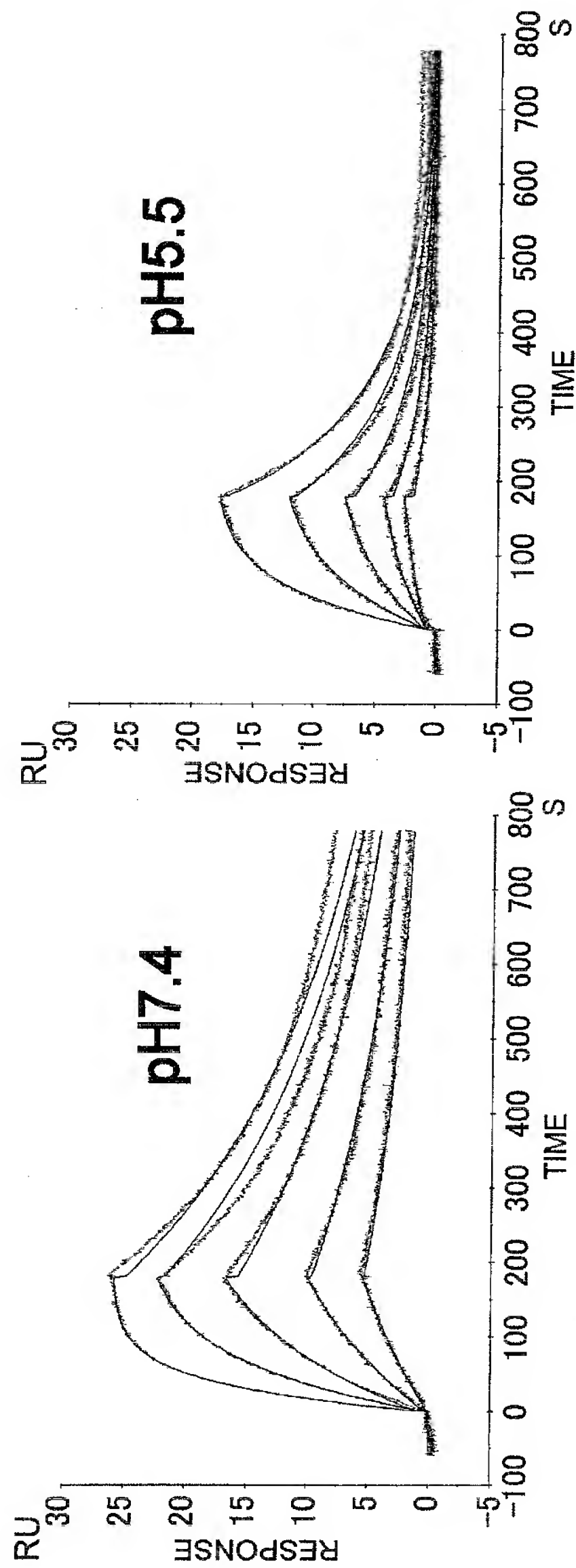


FIG. 26

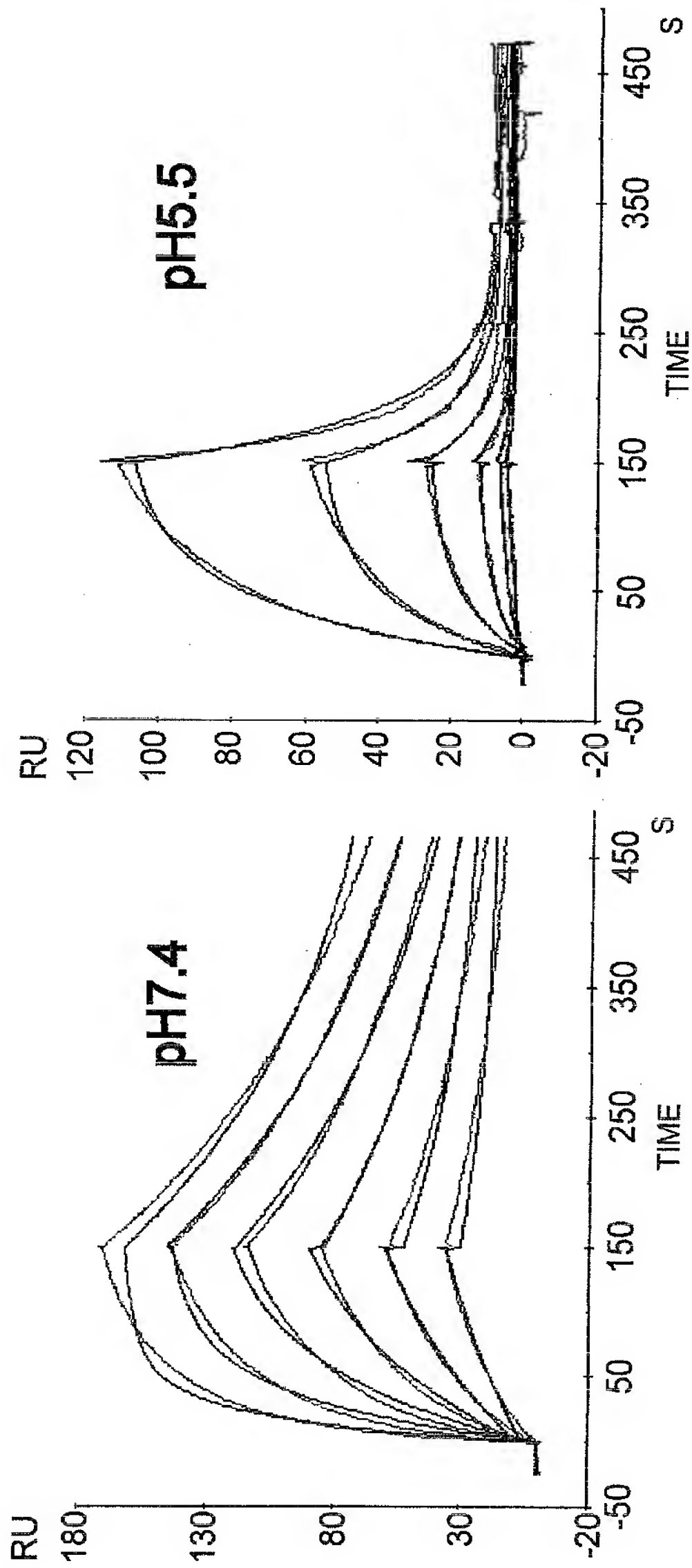


FIG. 27

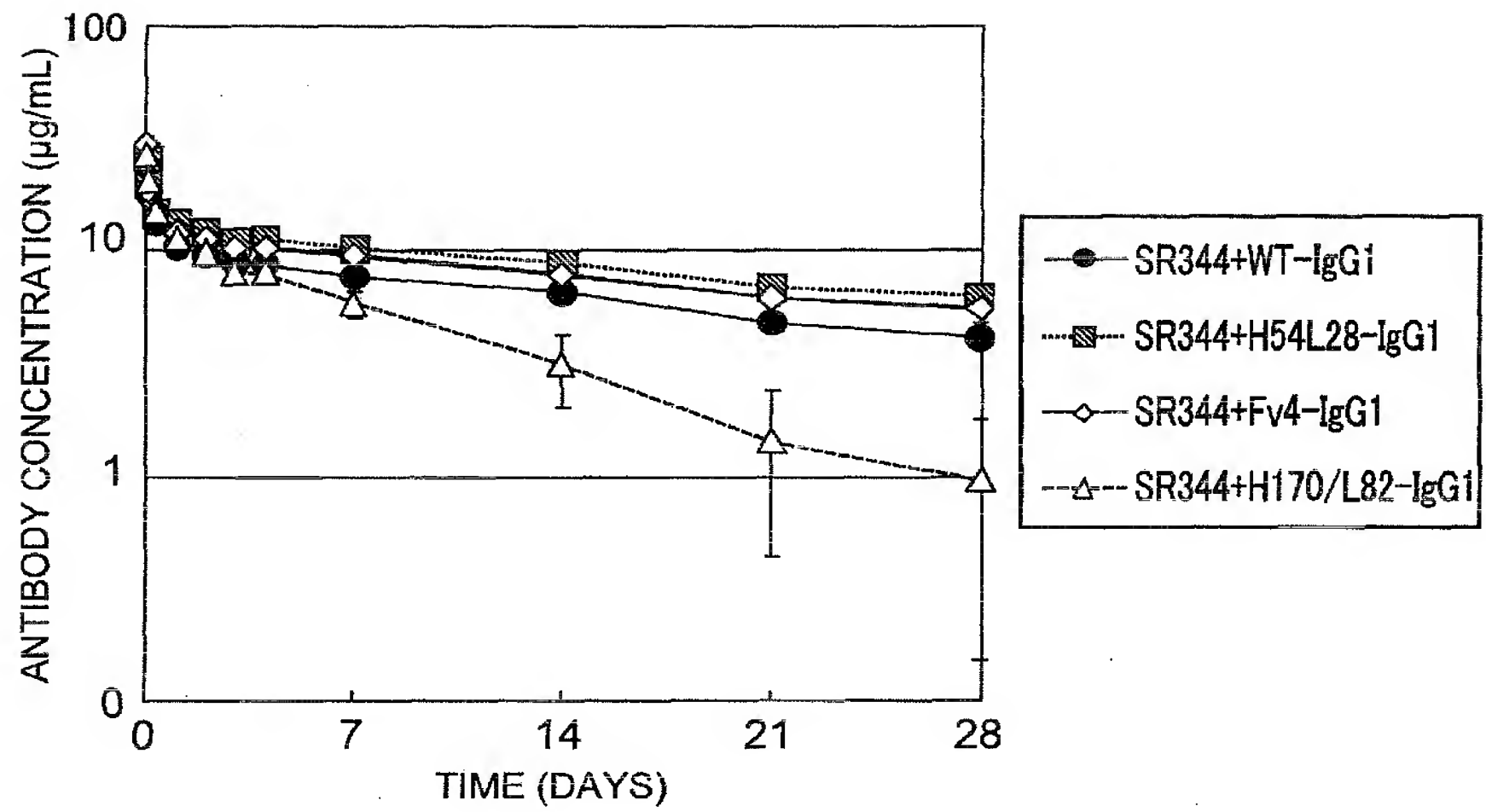


FIG. 28



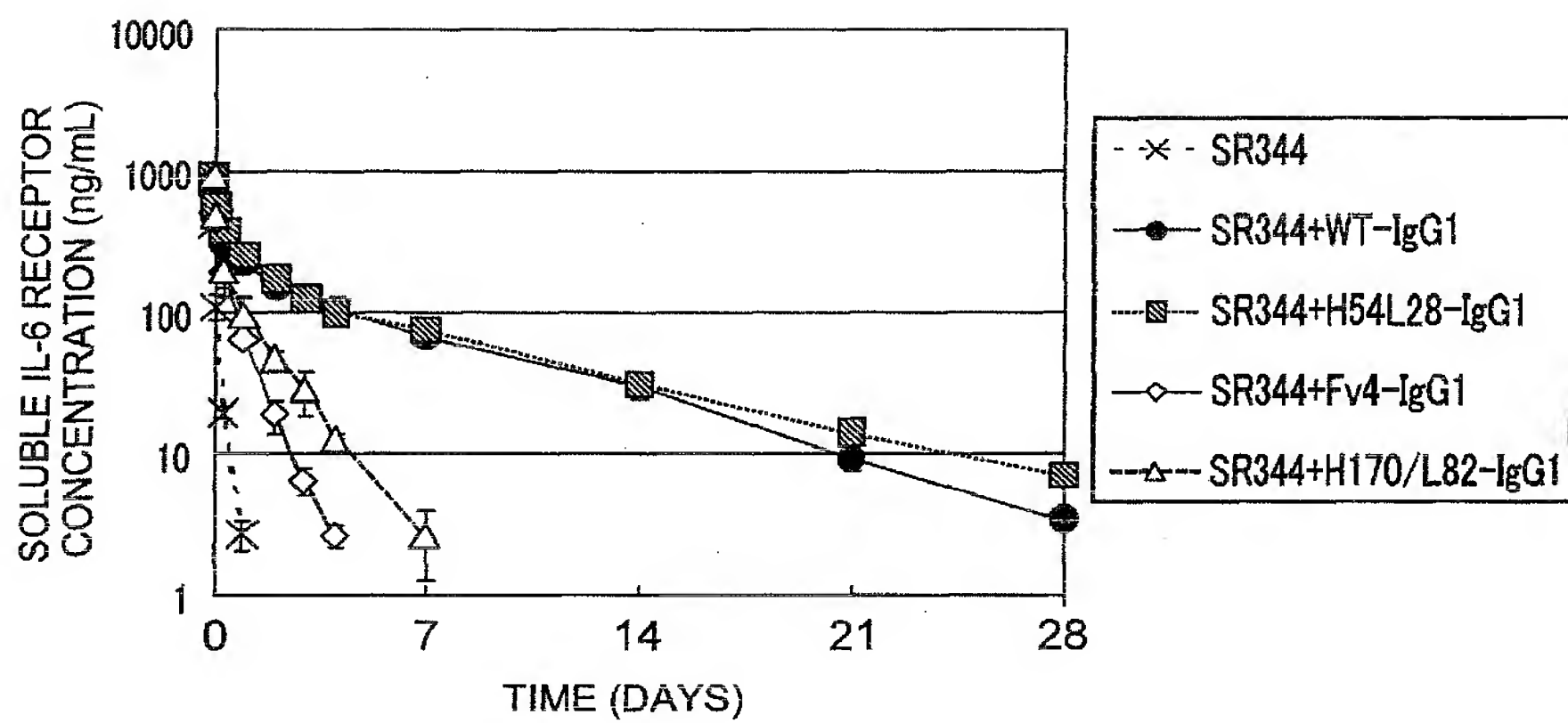


FIG. 29

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2009/057309

## A. CLASSIFICATION OF SUBJECT MATTER

C07K16/28(2006.01) i, A61K39/395(2006.01) i, C12P21/08(2006.01) i, G01N33/15(2006.01) i, G01N33/50(2006.01) i

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K16/28, A61K39/395, C12P21/08, G01N33/15, G01N33/50

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Jitsuyo Shinan Koho	1922-1996	Jitsuyo Shinan Toroku Koho	1996-2009
Kokai Jitsuyo Shinan Koho	1971-2009	Toroku Jitsuyo Shinan Koho	1994-2009

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

JSTPlus/JMEDPlus/JST7580 (JDreamII), WPI

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
<u>X</u> A	ITO, W.et al. The His-probe method: effects of histidine residues introduced into the complementarity-determining regions of antibodies on antigen-antibody interactions at different pH values. FEBS Lett., 1992, Vol.309, No.1, p.85-88	<u>1-8, 28-50</u> 9-27
A	MARTIN, W.L.et al. Crystal Structure at 2.8Å of an FcRn/Heterodimeric Fc Complex: Mechanism of pH-Dependent Binding. Mol.Cell., 2001, Vol.7, p.867-877	1-50

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search  
22 April, 2009 (22.04.09)Date of mailing of the international search report  
07 July, 2009 (07.07.09)Name and mailing address of the ISA/  
Japanese Patent Office

Authorized officer

Facsimile No.

Telephone No.

Form PCT/ISA/210 (second sheet) (April 2007)

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2009/057309

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

The inventions of claims 1-50 include the following three inventions: an invention relating to an antigen-binding antibody which has a lower binding activity at pH 5.8 than that at pH 7.4 as claimed in claims 1 and 9-14; an invention relating to a method involving the step of substituting an amino acid residue in an antigen-binding molecule by a histidine residue or inserting a histidine residue into the antigen-binding molecule as claimed in claims 18-23; and an invention relating to a method for the screening or production of an antigen-binding molecule having a higher binding activity at a first pH value than that at a second pH value as claimed in claims 28, 30-32 and  
(continued to extra sheet)

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**  
the

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☒ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (April 2007)

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2009/057309

Continuation of Box No.III of continuation of first sheet (2)

43-46.

Further, at least the method for producing an antibody having a reduced antigen-binding activity under acidic conditions by inserting a histidine residue is known (see FEBS Lett., 1992, Vol.309, No.1, p.85-88). Therefore, the above-stated three inventions share no common special technical feature, and cannot be regarded as as being so linked to one another as to form a single general inventive concept.

## REFERENCES CITED IN THE DESCRIPTION

*This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.*

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